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Award Number: DAMD17-94-J-4313

TITLE: Characterization of CTL Recognized Epitopes on Human Breast Tumors

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REPORT DATE: October 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Final (19 Aug 94 - 18 Sep 00)

4. TITLE AND SUBTITLE
Charcterization of CTL Recognized Epitopes on Human Breast Tumors

5. FUNDING NUMBERS
DAMD17-94-J-4313

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Studies supported by this grant lead to identification of three novel antigens presented by breast cancer cells, which are recognized by cytotoxic T lymphocytes (CTL). These tumor antigens are (1) HER-2 (HER-2/neu proto-oncologene product,) (2) folate binding protein (FBP), folate receptor - α, and (3) amino enhancer of split (AES), a transcription factor associated with the Notch complex. These epitopes associated with these antigens were identified in association with HLA-A2. HLA - A2 is expressed in the majority (45-50%) of the North American population. Therefore these epitopes are important for targeting adoptive and vaccine therapies. The ability of peptides mapping these epitopes to activate lymphocytes demonstrated that these epitopes are immunogenic and can be incorporated in vaccines against breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	89
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Constantin G. Francis 01/02/200

PI - Signature Date

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Final Report

Award Number: DAMD 17-94-3-4313

The major goal of this grant was to study the existence of immunity to breast cancer in humans. The major objectives of this grant were to identify novel tumor antigens in breast cancer, to identify the immunodominant epitopes recognized by cytotoxic lymphocytes in breast cancer, to identify the responses induced by these epitopes (cytolysis and cytokines), to identify approaches to induce responses to these epitopes in healthy donors and cancer patients, as well as the receptor composition of the T cells responding to these epitopes. Studies in ovarian cancer were performed in parallel, since the original model and availability of indicator tumor cells as well as of effectors of defined specificity allowed a better correlation and confirmation of the responses.

The contract has been extended to September 2000. The last two reviews 1998-1999 indicated that this investigator not only fulfilled, but exceeded the goals of this proposal, and the research was extremely successful.

The key accomplishments of this research are:

(1) Identification and characterization of three novel tumor Ag. They are: HER-2, folate binding protein, and aminoenhancer of split of the Notch complex. It should be mentioned that most cellular therapy approaches by CTL used the ground work prepared by this proposal at its outcoming publications. Thus, the DOD support had a far reaching significance, which exceeded expectations.

(2) Studies on folate binding protein will have a particular relevance in the future, because of the use of metotrexate as chemotherapeutic agent. Currently the results with FBP are evaluated independently at preclincial level, to determne the applicability for vaccine.

(3) All together HER-2, FBP, and AES constitute the nucleus for development of multi-epitope vaccines. An additional point of interest for future studies is the applicability of these antigens for breast tumors at different stages of aggressivity and differentiation. AES may be important for poorly differentiated tumors of HER-2 negative phenotype. HER-2 for HER-2$^+$ tumors while FBP may be of use in a later stage (FBP increases after chemotherapy).

(4) Tumor cells expressing MHC-I and HLA-A2 were associated with the over growth of drug-resistant tumors, which were susceptible to cytotoxic T-lymphocytes attack. Investigation of the mechanisms for this sensitivity revealed that one potential mechanism is the formation of unstable tumor antigens which degrade at a faster rate than the tumor Ag present in the cell not treated with chemotherapeutic drugs. This aspect was completed this year and the relevant publication is currently in print (Molecular and Cellular Biochemistry). Additional aspects of this mechanisms are the post-translational modifications of the tumor Ag, in terms of phosphorylation made the influence of HER-2 receptor agonists. For example, for HER-2, the agonists are EGF and NDF (new differentiation factor). There are novel aspects of tumor immunogenicity which open

new directions of research and treatment. Tumors become antigen presenting cells (APC) is treated with various agents which enhance the presentation of the Ag as well as the expression of HLA-molecules.

(5) **(a)** Studies on antigenicity of the tumor Ag, demonstrated that the tumor Ag are weak and partial agonists. The partial agonism manifest as a weak ability to induce the cytokine IFN-γ, and the chemokine IP-10 (anti-angiogenic) increased IFN-γ and IP-10 could be achieveld by low amounts of IL-12 (100-200 pg/ml). Notably, IP-10 induction by tumor Ag, does not require IFN-γ, but it is potentiated by IL-12. This should be one of the earliest detectable effects of T-cell interaction with the tumor Ag.

The ability of low doses of IL-12 (100 pg/ml/g = 100 ng/kg) to costimulate the tumor Ag effects are significant for tumor vaccine development: A clinical trial is being prepared by Dr. James A. Murray, to obtain the NIH approval. It is my understanding that the previous handling of high dose IL-12 generated concerns.

(6) **(b)** The "repair/correction" of the agonistic activity of the tumor Ag by IL-12 with regard IFN-γ, it is not sufficient to correct the inability of the tumor Ag to induce cytolytic effector function at priming. An extensive study using ten (10) healthy donors recently completed revealed that only a fraction (40-50% of the health donors responded to the CTL epitope from HER-2 (E75). Is this the fraction of responders to HER-2 vaccine based on E75 in the population? Is the 50% the fraction of non-responders to the wild-type tumor Ag, for various reasons? These questions will be addressed in the future.

(7) To address how tumor immunogenicity can be improved various approaches were used: APC: dendritic cells ± TNF-α (as maturing agent) vaccinia vectors: imunogenicity was obtained only with replicating vaccinia. This is acceptable in vitro, but cannot be used for *in vivo* studies because the patients are immunocompromised.

(8) The limitations in immunogenicity by DC, although DC were much better APC than PBMC and nonreplicating vaccinia vectors, lead to novel approaches for enhancement of immunogenicity using enhancer agonists. The enhancer agonists were generated by introducing mutations in the Ag-sequence.

(9) A large number of mutated E75 peptides were generated aiming to induce TCR-enhanced agonism, and not the traditional enhanced binding ability to HLA-A2 molecules. A previous study also supported by this grant, identified the basic principles for modulation of TCR antigenicity. Research in this direction identified one agonist designated F42, and generated by a mutation serine → lysine in position 5, which was effective ininducing CTL specifically recognizing E75. These CTL were induced from both healthy donors and breast cancer patients. The TCR-type-enhancer-peptides will be significant for the further development of cancer vaccines in breast cancer.

(10) An immediate consequence of the studies are:

(1) A clinical vaccine trial in M. D. Anderson Cancer Center by Drs. James L. Murray (Breast Clinic/Bioimmunotherapy) and Dr. Andrzej Kudelka (Department of Gynecologic Medical Oncoloty)

(2) A clinical vaccine trial at Walter Reed Army Institute of Research and the Department of Surgery of the Uniformed Armed Services, by Dr. George E. Peoples and Dr. Bryan Fisk, both formerly in the laboratory of this grantee.

Reportable outcomes: The reportable outcomes of this study are presented in detail in the publications listed below:

Publications

Fisk, B., Blevins, T.L., Wharton, J.T. and **Ioannides, C.G.** Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor specific CTL lines. J. Expt. Med. 181: 2109-2117, 1995.

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Anderson, L. D. Jr., Hudson, J. M., Savary, C. A., Fisk, B, Gershenson, D. M., and **Ioannides, C.G.** HER-2/neu peptide specificity in the recognition of HLA-A2 by natural killer cells. Cancer Immunol Immunother, 48(7):401-410, 1999.

Peoples, G.E., Anderson, B.W., Lee, T. V., Murray, J.L., Kudelka, A.P., Eberlein, T.J., Wharton, J.T., and **Ioannides, C.G.** Vaccine implications of folate binding protein in epithelial cancers. Clin. Cancer Res., 5:4214-4223, 1999

Kim, D., Lee, T. V., Castilleja, A., Anderson, B. W., Kudelka, A. P., Murray, J. L. Sittisomwong, T., Wharton, J. T., Kim, J., **Ioannides, C. G.** Folate binding protein peptide 191-199 presented on dendritic cells can stimulate CTL from ovarian and breast cancer patients. Anticancer Res, 18:2907-2916, 1999.

Lee, T.V., Castilleja, A., Peoples, G.E., Kim, D-K, Murray, J.L., Gershenson, D. M., and **Ioannides, C. G.** Secretion of CXC chemokine IP-10 by peripheral blood mononuclear cells from healthy donors and breast cancer patients stimulated with HER-2 peptides, J. Interf. Cytokine Res., 20,391-401, 2000.

Lee, T. V., Anderson, B. W., Peoples, G.E., Castilleja, A., Murray, J.L., Gershenson, D. M. and **Ioannides, C.G.** Identification of activated tumor-Ag-reactive $CD8^+$ cells in healthy individuals, Oncology Reports, 7:455-466, 2000.

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Abstracts

Blevins, T. L., Fisk, B., Wharton, J.T., Kavanagh, J.J., **Ioannides, C.G.,** and Kudelka, A.P. T Cell proliferating responses to HER-2 peptide analogs in ovarian cancer patients. The FASEB Journal. 9(4): A800, 1995.

Fisk, B., DaGue, B., Seifert, W. E., Lambris, J. D., Papayannopulos, I., Kudelka, A., Wharton, J. T., and **Ioannides, C. G.** Ovarian cytotoxic T lymphocytes can recognize common peptides presented by HLA-A2+ tumors. The FASEB Journal, 10(5): A1467, 1996.

Ioannides, C. G., Murray, J. L., and Wharton, J. T. Recognition of breast and ovarian cancer antigens by T lymphocytes. 3rd International Conference on Engineered Vaccines for Cancer and AIDS, October 9-14, 1996, Hilton Head Island, South Carolina.

Anderson, B.W., Swearingen II, B.J., Wharton, J.T., and **Ioannides, C.G.** Primary generation of human antitumor CTL by a HLA-A2 restricted HER-2 peptide. Proceedings of AACR, Vol. 38, A1606, p239, 1997.

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Murray, J.L., Babcock, B., Anderson, B., Kudelka, A., Wharton, J.T. and **Ioannides, C.G.** Ovarian and breast cytotoxic lymphocytes can recognize peptides from the AES protein of the Notch complex. Proceedings of the AACR,39:, A1051, p 154, 1998.

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Anderson, B., Lu, J., Ibrahim, N., Hortobagyi, G., Brewer, H., Przepiorka, D., **Ioannides, C.G.,** Grabstein, K., Cheever, M., and Murray, J. Immunomodulating effects of a HER2/neu derived HLA-A2 specific peptide E75 combined with GM-CSF in patients with metastatic breast and ovarian cancer. Proceedings ASCO, 18, A1691, 1999.

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Lee, T. V., Castilleja, A., Peoples, G. E., Kim, D. K., Gershenson, D. M., **Ioannides, C. G.** Secretion of CXC chemokine IP-10 by T cells from healthy donors and breast cancer patients stimulated with HER-2 peptides. 17th Annual Texas Immunology Conference, October 18, 1999.

Note: All these publications were supported by this grant. The grantee, regrets that in some instances the support was not acknowledged by unwanted errors. If an affidavit is necessary we will be more than happy to provide one.

Key Personel Involved in this Research:

1. Bryan Fisk
2. Tracy L. Blevins
3. J. Michael Hudson
4. Brett W. Anderson
5. Agapito Castilleja
6. Ben Babbock
7. Tom V. Lee
8. Nancy E. Ward
9. Bruce Swearingen

In conclusion, the grantee wishes to express his gratitude and appreciation to the DOD Breast Cancer program. The vision of this program and the outstanding support received from Dr. Patricia Modrow, Ms. Judy Pawlus, as well as from the reviewers, made the accomplishment of this goal possible. Their support was essential for taking this study from the hypothesis level to the clinical treatment level.

Drs. Murray, Peoples, Fisk and O'Brian join the undersigned in expressing their appreciation for this initiative which revolutionized the vaccine approaches to breast cancer.

Thank you very, very much.

Constantin G. Ioannides

Constantin G. Ioannides, Ph.D.
Professor

JOURNAL OF INTERFERON AND CYTOKINE RESEARCH 18:529–536 (1998)
Mary Ann Liebert, Inc.

Growth and Antigen Recognition of Tumor-Infiltrating Lymphocytes from Human Breast Cancer

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ANDREZJ KUDELKA,[3] EVA SINGLETARY,[4] J. TAYLOR WHARTON,[1]
and CONSTANTIN G. IOANNIDES[1,5]

ABSTRACT

In the present study, we isolated tumor-infiltrating lymphocytes (TIL) from 21 primary solid tumors and tumor-associated lymphocytes (TAL) from 9 malignant effusions, respectively, of breast cancer patients. Significant proliferation and expansion of T cells was observed in 23 of 30 distinct samples. TIL were isolated from primary tumors by either enzymatic digestion or mechanical disruption. The TIL cultures were initiated using OKT3 mAb in the presence of moderate concentrations (25–50 U/ml) of IL-2, followed by 100 U/ml of tumor necrosis factor (TNF)-α. TAL were not stimulated with OKT3 mAb, but all were successfully expanded in culture in the presence of IL-2 alone or together with TNF-α. Seven of nine distinct TAL grew in culture as predominantly $CD4^+$ lines. In contrast, only 14 of 21 (66%) of primary breast TIL expanded in culture and were predominantly of $CD8^+$ phenotype. Autologous tumor lysis was observed in seven of eight cases tested. Only one of the four TIL tested and one of the four TAL tested preferentially lysed autologous tumor. HER-2 peptide E75 (369–377) was recognized by two TIL lines of the five primary TIL tested and three of the four TAL tested. This suggests that E75 may be recognized by primary breast tumors. This may be of interest in developing vaccine strategies for therapeutic management of breast cancer.

INTRODUCTION

INFILTRATION OF HUMAN TUMORS with lymphocytes is believed to indicate an immune response to tumor, as shown by a large body of studies in melanoma, renal cell carcinoma, and ovarian carcinoma. Similarly, T cells constitute the dominant leukocyte population associated with primary breast tumors. Of these, $CD8^+$ cells are present in tumor infiltration sometimes in higher percentage than in peripheral blood. Correlation between HLA-DR expression in $CD8^+$ tumor-infiltration lymphocytes (TIL) and MHC-I and MHC-II expression on the breast tumor suggests a local immune reaction that cannot expand or eradicate the primary carcinoma.[1] Strong evidence for local activation of mononuclear cells *in situ* was exemplified by expression of cytokines and cytokine genes.[2,3] Selective expression of early activation markers was found in breast TIL associated with depressed levels of interleukin-2 (IL-2) and IL-2R, suggesting that TIL may be in a relatively anergic state.[3,4] These results are indicative of the presence of antigenic molecules for T cells at the primary tumor site.

Despite the high incidence of breast cancer, there are few studies on the immune recognition and antigens of breast tumor. There are also few studies on the feasibility of propagation of TIL from primary breast tumors. This is compounded by difficulties in isolation of breast TIL from small tumors (<0.5 cm). Evidence that breast TIL can recognize autologous tumor has been reported.[5–10] Breast TIL propagated in high concentrations of IL-2 plus lymphokine-activated killer (LAK) cell supernatant infrequently exhibited preferential lysis of autologous tumor but more frequently exhibited specific Th1 cytokine secretion.[6,8–10] Reversal of T cell anergy *in vitro* using OKT3 mAb and repeated stimulation with autologous tumor in the presence of moderate concentrations of IL-2 resulted in a high proportion of tumor-specific cytotoxic T lymphocytes (CTL) in some instances.[5,8] As this approach is dependent on the availability of autologous tumor, an alternative approach to

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breast CTL expansion is to use antigen (Ag) recognized by these breast TIL to propagate tumor-specific CTL. Using different methods, breast TIL have been isolated that recognized MUC-1, MAGE-1, and a HER-2 peptide designated GP2 (654–662).[11–13] The Ag specificities recognized by these CTL may not always be present on the autologous tumor at the time of collection, or the specific CTL frequency may be low (in these patients, the disease progresses). However, they may reflect existent responses to sensitizing Ag expressed earlier by the tumor. The identification of several CTL epitopes on HER-2[14] presented by HLA-A2 provide a model in which these questions can be addressed.

The objectives of this study were twofold: (1) to determine if breast TIL can be expanded from small primary breast tumors and (2) to determine if breast TIL and tumor-associated lymphocytes (TAL) can lyse breast tumors and recognize HER-2 peptides defining CTL epitopes. Because TIL culture in high concentrations of cytokines may lead to activation of different effector populations, such as LAK cells, and repeated stimulation with autologous or HLA-matched tumor may lead to amplification of restricted specificities present on the stimulators, we used an alternative approach for T cell propagation. This consisted of coculture of breast TIL in the initial presence of autologous tumor (when available) and OKT3 mAb and moderate concentrations of IL-2 (25–50 U/ml). We found that breast TIL from small primary tumors isolated by enzymatic digestion or only mechanical disruption could be expanded *in vitro*. HER-2 peptide, E75 (369–377) previously shown to be recognized by ovarian TAL was also recognized by a number of breast TIL and breast TAL.

MATERIALS AND METHODS

Isolation and culture of breast TIL and TAL

Fresh primary breast tumors were obtained from 21 patients. TIL were isolated after enzymatic digestion for 4 h at 37°C, followed by separation of tumor cells from lymphocytes over 75%–100% Ficoll gradients.[10] The lymphocyte-enriched fraction containing 10%–20% tumor cells was cultured in RPMI medium with 10% fetal bovine serum (FBS), 40 μg/ml gentamicin (complete RPMI), and 50 U/ml IL-2 (Cetus) in 24-well plates (Costar, Cambridge, MA). In case of recovery of a small number of cells, TILs were grown in 48-well plates (Becton-Dickinson, Franklin Lakes, NJ) in medium with IL-2 at an initial cell density of 10^6/ml. In many instances, breast TIL from primary tumors showed slow growth during the first week in culture. To facilitate their expansion, they were cultured initially on plates coated with OKT3 mAb as described[8] in complete RPMI medium supplemented with 50 U/ml IL-2 and 100 U/ml tumor necrosis factor-α (TNF-α).[15]

When the tumor specimen was small ($\leq$0.5 cm) enzymatic digestion, even for a limited period of time, led to poor recovery of TIL. These cells could not be expanded in cultures. We, therefore, used an alternative approach for breast CTL expansion. This approach is based on the use of small tissue fragments for the growth of TIL.[15] The tumor tissue was cut in pieces ($\leq$1.0 mm) that were cultured in complete RPMI medium with 25 U/ml IL-2 and 100 U/ml TNF-α for the first 16 h. Afterward, OKT3 mAb (10 ng/ml) was added to the cultures. After 2–3 days, leukocyte migration out of the tumor pieces was observed. Tumor pieces were dispersed by pipetting up and down. The mixture was allowed to settle for 30–40 sec, and the cell suspension was replated. This procedure provided better yields of recovery than enzymatic digestion and was used for TIL-10 through TIL-14. Malignant effusions were obtained from nine patients (pleural effusions in five and ascites in four patients). Isolation of TAL was performed as described.[16] Briefly, after pelleting, the cells were resuspended in serum-free medium, layered, and centrifuged over a 75%–100% Ficoll gradient. Purified lymphocytes were cultured in complete medium without OKT3 mAb. $CD8^+$ cells were isolated on specific mAb-coated plates as described.[14]

Phenotype analysis

Expression of HLA-A2 on breast TAL and TIL and of HER-2 on tumors was determined using mAb BB7.2 (American Type Culture Collection, Rockville, MD) and Ab-2, specific for the extracellular domain of HER-2 (Oncogene Science, Manhasset, NY) as described. Briefly, the cells were incubated with primary antibody for 30 min at 4°C, followed by fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Beckton Dickinson, San Jose, CA). Expression of CD3, CD4, and CD8 molecules on TIL and TAL was determined by direct immunofluorescence using OKT3, OKT4, and OKT8 mAb conjugated with FITC (Ortho Diagnostic, Raritan, NJ). Surface antigen expression was determined using a FACScan flow-cytometer (Beckton Dickinson) with a log amplifier.

Synthetic peptides

HER-2 peptides were prepared by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid-phase method and purified by HPLC. The designation of these peptides follows that used in our previous studies: E75 (HER-2, 369–377) KIFGSLAFL; F53 based on the sequence of GP2 (HER-2, 654–662) IISAVVGIL; E90 (HER-2, 789–797); E89 (HER-2, 851–859) VLVKSPNHV; C85 (HER-2, 971–979) ELVSEFSRM. Identity of peptides was established by amino acid analysis. The purity of peptides was more than 95%.

Cytotoxicity assays

CTL activity was measured in [^{51}Cr] release assay as previously described.[14,16] Where specific lysis was low (5%–10%) in 4–5-h CTL assays, incubation of effectors with targets was continued, and the release of [^{51}Cr] was determined again the next morning. Autologous and freshly isolated allogeneic tumors as well as the breast tumor lines SKBR3 and SKBR3.A2 were used as targets. SKBR3.A2 cells express a transfected HLA-A2 gene. The original SKBR3.A2 transfectant (HLA-A, 11, B18, 40), kindly provided by Drs. Mary Disis and Martin Cheever (University of Washington, Seattle), was subjected to additional rounds of selection with the antibiotic G418 (Gibco, Life Science). The resulting cells were 100% HER-2^+, 61.6% HLA-$A2^+$, mean channel fluorescence (MCF) = 466, compared with parental SKBR3 cells, which were 5.2% HLA-$A2^+$ (MCF = 29), and control C1R.A2 cells, which were 95% HLA-$A2^+$ (MCF = 430). To study recognition of HER-2 peptides,

we used T2 cells as indicators.[16] Control cells were made with T2 cells incubated with effectors in the absence of synthetic peptides.

RESULTS

Growth and T cell phenotype of TIL and TAL from breast cancer

TIL and TAL were propagated *in vitro* with moderate concentrations of IL-2 (25–50 U/ml). Lymphocytes from both malignant effusions and solid primary tumors exhibited significant expansion in culture over time. TIL were obtained from relatively small pieces of tumor (<2 cm in diameter). After 1–2 weeks in culture, tumor cells and other nonlymphoid cells disappeared. The culture was continued as necessary to obtain lymphocytes for further studies (usually 3–4 weeks). Overall, of 14 distinct primary breast tumors processed by enzymatic digestion, 9 distinct TIL (64%) were successfully established in culture. These TIL are listed in Table 1. The doubling time for four TIL isolated by enzymatic digestion was 3 days, for one 4 days, for three 5 days, and for the remaining one >7 days. In contrast, for most TAL, the doubling times were 3 days, and only for one 4 days. The fold expansion in the first 4 weeks ranged between 40-fold and 250-fold (data not shown). A similar pattern of rapid growth was observed for TIL isolated from tumor samples that were not subjected to enzyme digestion. As we could not determine the initial number of T cells in TIL processed without enzymatic digestion, we could not establish their doubling times. The large numbers of T cells present after 2 weeks in TIL-10-13 cultures suggested a vigorous growth in four of five cultures (except TIL-14).

Table 1. Phenotypic Characteristics of Breast TIL and TAL Cultures[a]

	Origin	HLA-A2	% Positive CD4	% Positive CD8
Tumor-infiltrating lymphocytes				
1	Primary	+	8	95
2	Primary	+	4	95
3	Primary	+	4	30
4	Primary	+	75	25
5	Primary	+	30	64
6	Primary	−	69	30
7	Primary	−	81	27
8	Primary	−	12	85
9	Primary	−	33	67
Tumor-associated lymphocytes				
1	Pleural effusion	+	65	35
2	Pleural effusion	+	60	36
3	Pleural effusion	+	66	33
4	Pleural effusion	−	74	33
5	Ascites	−	45	29
6	Ascites	−	*n*[b]	48
7	Ascites	−	73	20
8	Ascites	+	13	87
9	Pleural effusion	+	*n*	72

[a]The average doubling time for TIL and TAL from breast tumors determined for weeks 2 and 3 was 3 days excepting TIL-9 and TAL-4 (4 days), TIL-4 and TIL-8 (5 days), and TIL-5 (>7 days). TAL 1–4 and 9 were obtained from pleural effusions, whereas TAL-5–8 were from ascites.

[b]*n* indicates not determined.

Table 2. Surface Phenotype and Growth Characteristics of Freshly Isolated Breast TIL Separated Without Enzyme Digestion

TIL	HLA-A2	% Positive CD4+	% Positive CD8+	Days in culture	Cells ($\times 10^6$)[a]
10	+	16	89	14	10.5
11	+	8	95	14	12.6
12	−	29	59	21	34.0
13	+	4	61	7	7.4
14	+	37	46	11	4.7

[a]Breast tumor samples <0.5 cm were cut into small pieces and cultured in complete RPMI 1640 containing 25 U/ml IL-2 and 100 U/ml TNF-α. OKT3 mAb was added to the culture after 24 h, and the culture was continued for the indicated number of days. The numbers of lymphocytes could not be determined in the original sample.

We succeeded in propagating five of seven distinct primary breast TIL isolated by mechanical disruption of the tumor, followed by OKT3 mAb (71%) (Table 2). This included TIL-12, TIL-13, and TIL-14, which could not be propagated after enzymatic digestion. Taking into consideration that enzymatic digestion results in low cell yields and may lead to significant damage of infiltrating lymphocytes, this approach may be useful for isolation of TIL from small breast samples or needle biopsies. The T cell phenotype of the TIL was determined together with the expression of HLA-A2 (Tables 1 and 2). Nine of 14 (60%) cultures were HLA-A2$^+$. Overall, CD4$^+$ T cells predominated in 3 TIL cultures (21%), whereas CD8$^+$ cells were more numerous in 9 cultures (64%). In two cultures, both CD4$^+$ and CD8$^+$ cells failed to reach the level of 50%. A different T cell phenotype was observed for breast TAL. Seven of nine proliferated as predominantly CD4$^+$ cells. Five of nine TAL samples (50%) were HLA-A2$^+$. This is in the HLA-A2 range observed in the North American population.

Target recognition by breast TIL and TAL

Autologous tumors in sufficient numbers and of sufficient purity to study target recognition were obtained from four primary breast cancers (Nos. 1, 2, 3, 4) and four patients with metastatic tumors (Nos. 1, 2, 3 and 6). Overall, one of four HLA-A2$^+$ breast TIL and one of four breast TAL tested showed preferential recognition of autologous tumors. The specificity control targets consisted primarily of the tumors autologous with TAL-1 (HLA-A2$^+$) and TAL-6 (HLA-A2$^-$), respectively, which were lysed at low levels by the autologous TIL, and the breast lines SKBR3.A2$^-$ and SKBR3.A2$^+$. Only TIL-3 and TAL-3 showed preferential lysis of autologous tumors (Fig. 1). TAL-3 showed similar levels of lysis of autologous tumor and

of the SKBR3.A2 line but lower levels of lysis of HLA-A2$^-$ targets, suggesting the presence of a shared Ag between autologous tumor and SKBR3.A2 cells.

TIL-1 showed borderline lysis (≤5%) of autologous tumor at an E:T ratio of 20:1. TIL-2 showed nonspecific tumor lysis. TIL-4 lysed the autologous tumor minimally. Three other TIL (Nos. 6, 8, and 9), for which the autologous tumor was not available, were tested in the same experiment against a panel of three breast tumors and the NK target K562. TIL-6 and TIL-8 showed very high lysis of K562 cells (>50%) and low lysis of all the tumor targets (<20% at E:T = 20:1) (data not shown). We tentatively concluded that these TIL do not exhibit specific autologous tumor lysis. TIL-9 showed significantly lower lysis of K562 (<40% in the same conditions) suggesting that specific tumor lysis may be possible.

Of two breast CTL lines (TIL-4 and TAL-2) that lacked specificity for autologous tumor, TIL-4 lysed minimally the autologous tumor and marginally two breast targets (allogeneic tumor and SKBR3) but showed significantly higher lysis of both K562 and Daudi (MHC-I negative) cells, suggesting that they express an NK/LAK-like activity (Fig. 3A). TAL-2 lysed the autologous tumor (A2$^+$), the freshly isolated allogeneic tumor associated with TAL-1 (A2$^+$), and in some experiments SKBR3 marginally (Fig. 2). In contrast, these breast CTL showed higher lysis of LAK targets but not of the tumor targets.

Because these results suggest that tumor-specific CTL may be either present in low numbers or anergic or both, we examined the effect of stimulation with OKT3 mAb in the presence of IL-2 on the expression of autologous tumor lysis by TAL-1. The results are shown in Figure 3. Stimulation with OKT3 mAb of TAL-1, containing both CD4$^+$ and CD8$^+$ cells, led to a minimal increase in target lysis. OKT3 stimulation was most effective in eliciting the TAL-1 lytic function when separated CD8$^+$ cells were used (Fig. 3, d). The biggest difference in lysis between the autologous tumor and K562 was seen in the long CTL assay (20 h), indicating, as previously suggested, low-frequency tumor-specific CTL.[17]

Breast TIL and TAL recognize CTL epitopes formed by HER-2 peptides

Tumor-reactive CTL have been reported to recognize peptides derived from tumor Ag. Studies have shown that breast CTL can recognize (1) the core peptide from the MUC-1 in both MHC-restricted and nonrestricted fashion,[18] (2) the MAGE-1 melanoma Ag,[13] and (3) a HER-2 epitope defined by the peptide GP2 (654–662).[12] Because the HER-2 peptide E75 (369–377) was found to be immunodominant in ovarian cancer patients, we tested recognition of E75 by five breast TIL lines and four breast TAL lines. The results are shown in Figure 4. TIL-1 and TIL-2 significantly recognized E75. TIL-3, TIL-10, and TIL-11 did not recognize E75. This indicates that E75 is recognized by two of five CTL from primary breast tumors. TIL-10 and TIL-11, which did not recognize E75, recognized marginally E90 (HER-2, 789–797) and C85 (HER-2, 971–979) (data not shown). To address the question of whether breast TAL recognized E75, we tested recognition of this peptide by TAL-1, TAL-2, TAL-3, and TAL-9. These TAL were HLA-A2$^+$ and associated with HER-2^{hi} breast tumors. The results are shown in Figure 4. TAL-1, TAL-2, and TAL-9 recognized E75, but TAL-3 did not. These results indicate that E75 was recognized by three of four of the breast TAL lines. Overall, among five TIL and four TAL lines studied, five recognized the peptide E75, suggesting that it may represent a shared antigen in breast cancer.

Comparison of the CTL activity levels indicates that these

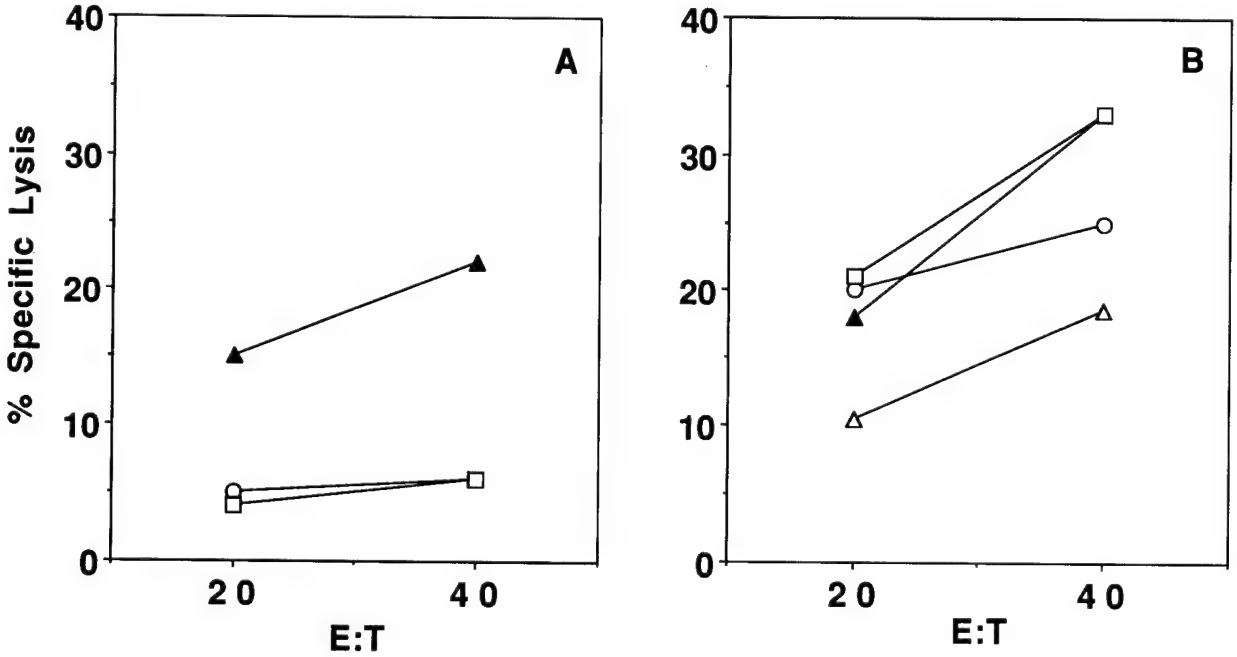

FIG. 1. Target recognition by (A) TIL-3, (B) TAL-3, (▲) autologous tumor, (△) HLA-A2$^-$ freshly isolated allogeneic tumor, (○) SKBR3 (HLA-A2$^-$), (□) SKBR3.A2 (HLA-A2$^+$). TIL-3 lysed significantly K562 cells (≥5%) only at 40:1 E:T.

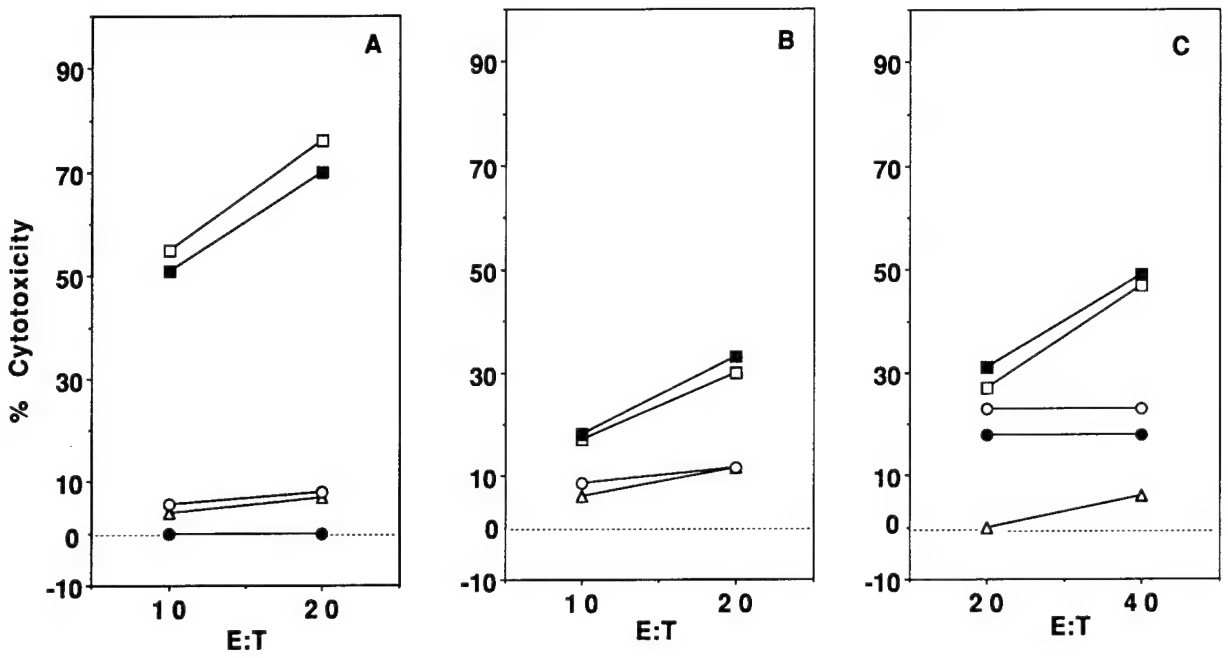

FIG. 2. (A) Cytolytic activity of TIL-4 isolated $CD8^+$ cells after 5 weeks in culture. Freshly isolated tumor (●) (□) K562, (■) Daudi, (○) tumor associated with TAL-1, (△) SKBR3. Results were determined in a 5-h [^{51}CR] release assay. Our representative experiment of two performed is shown. (B) Cytolytic activity of TAL-2 cultured for 3 weeks and stimulated with OKT3 mAb 1 week before the CTL assay. (C) TAL-2 re-stimulated with OKT3 mAb on week 3 cultured for a total of 5 weeks. (●) autologous tumor, (○) allogeneic, HLA-$A2^+$, HER-2^{hi} breast tumor, (□) K562, (■) Daudi, (△) SKBR3. The lysis of SKBR3.A2 (HLA-$A2^+$, HER-2^{hi}) was 0.0% at both 40:1 and 60:1 E:T ratios, suggesting that TAL-2 do not recognize HLA-A2-associated epitopes on this tumor.

TAL show either significantly lower recognition of autologous tumor than of HER-2 peptides (TAL-1, TAL-2) or higher specific recognition of autologous tumor but low recognition of HER-2 peptides (TAL-3). This suggests that the levels of expression of this peptide may be low and that breast CTL may recognize other Ag. Ongoing studies in our laboratory using TIL-1, TIL-2, TAL-1, and TAL-9 as indicators have identified another tumor Ag derived from the Notch receptor complex[19] (B. Babcock et al., unpublished observations).

DISCUSSION

The results presented in this study show a reproducible approach for expansion of breast TIL, with minimal intervention in terms of enzyme digestion, restimulation with autologous tumor/OKT3 mAb, and addition of exogenous cytokines. The nonenzymatic approach is complementary to enzymatic digestion. In fact, TIL-12, TIL-13, and TIL-14 were isolated from samples subjected in parallel to enzymatic and nonenzymatic processing, of which only the latter was successful. We succeeded in expanding in culture breast TIL from 14 patients and TAL from 9 additional patients. Of the established TIL cultures from primary tumors, 9 were propagated from TIL isolated by enzymatic digestion, and 5 were propagated without enzymatic digestion. TIL from 5 additional patients isolated by enzymatic digestion and from 2 patients isolated by mechanical disruption failed to grow in culture. There was a difference in the doubling times between the TIL and TAL populations. Most breast TAL showed fast proliferation, with doubling times of 3 days. In contrast, only half of breast TIL proliferated at this rate. For TIL and TAL expansion and expression of lytic function, the availability of tumor for repeated restimulation is viewed as a critical factor.[8] We observed significant T cell proliferation in 14 of 21 cases attempted (66%), including TIL from small tumors in which restimulation with autologous tumor cells could not be performed. Our results show that breast CTL-TIL can be propagated and expanded into large numbers even from small tumor samples after initial coculture with autologous tumor and stimulation with OKT3 mAb.

Fourteen breast TIL established in culture grew as predominantly $CD4^+$ or $CD8^+$ cells. This suggests that breast CTL-TIL can be successfully propagated *in vitro* in the presence of low doses of IL-2 and TNF-α, as shown by our previous studies with ovarian TAL.[18] IL-2 plus IL-12 was less effective than IL-12 plus TNF-α in propagating breast TIL (unpublished observations). This may be useful for both adoptive cellular therapies and tumor antigen identification for cancer vaccines.

The T cell phenotype of cultured TIL showed a different picture from the T cell phenotype of TAL with regard to predominance of $CD8^+$ or $CD4^+$ cells. Nine of 14 primary TIL expanded as predominantly $CD8^+$ lines (with $\geq$50% $CD8^+$ cells in the population), whereas only 2 of 9 TAL were predominantly $CD8^+$. As both TIL and TAL were cultured under the same conditions without regard of the source, the different outcomes in the phenotypes may suggest an active priming and

stimulation *in situ* of $CD8^+$ in primary tumors. The fact that the average of $CD8^+$ in the peripheral blood ranges between 25% and 33% suggests an active recruitment of $CD8^+$ cells at the primary tumor site, compared with the pleural effusion/ascitic TAL, where contamination from passenger lymphocytes may be higher.

The limited use of stimulation with tumor and OKT3 mAb for breast TAL expansion in the presence of moderate concentration of IL-2 led to breast CTL that expressed tumor lysis with higher frequency than reported in some studies[6,9,10] but lower than in other studies.[5,8] We found similar percentages (75%) of autologous tumor lysis by either TIL (3/4) or TAL (3/4). We found preferential autologous tumor lysis in only 1 of 4 breast TIL and 1 of 4 TAL tested (25%). The criteria for definition of specificity used in this study were similar to criteria used in our previous studies in ovarian cancer.[20] However, such criteria may suffer from inherent limitations because of the presence of shared Ag, the differences in target lysability, the lack of information on the HLA phenotype, and the limited amount of autologous tumor available.

Reports using cytotoxicity studies have established the presence among breast TIL and TAL of lymphocytes specifically recognizing autologous tumors. Baxevanis et al.[5] successfully expanded breast TIL from 10 patients and TAL from 2 patients after two cycles of stimulation with autologous tumor and moderate doses of IL-2; 8 of 12 TIL showed preferential lysis of autologous tumors. Specific recognition of autologous tumor was also documented by testing for cytokine secretion.[9] Dadmarz et al.[6] reported that a number of $CD4^+$ TIL recognized autologous tumor in a MHC class II restricted fashion. Specific recognition of $CD4^+$ T cells was shown by determining tumor-specific cytokine release.[6] Linehan et al.[8] successfully expanded breast TAL using low-dose IL-2 and repeated stimulation with OKT3 mAb and tumor from six of six specimens. These TAL showed preferential lysis of the autologous tumor.

The use of autologous tumor and OKT3 stimulation only at initiation of the culture may be useful to identify dominant epitope specificities of tumor-associated CTL *in situ*. The HER-2 peptides recognized by breast TIL may reflect Ag that have been stimulatory during tumor progression but either they were not sufficiently strong to elicit a curative response or novel tumor variants were selected. These variants may not express these Ag in sufficient amounts to sensitize CTL for killing or may express other Ag. It should be mentioned that all tumor Ag reported to date are recognized with low affinity.[16,21]

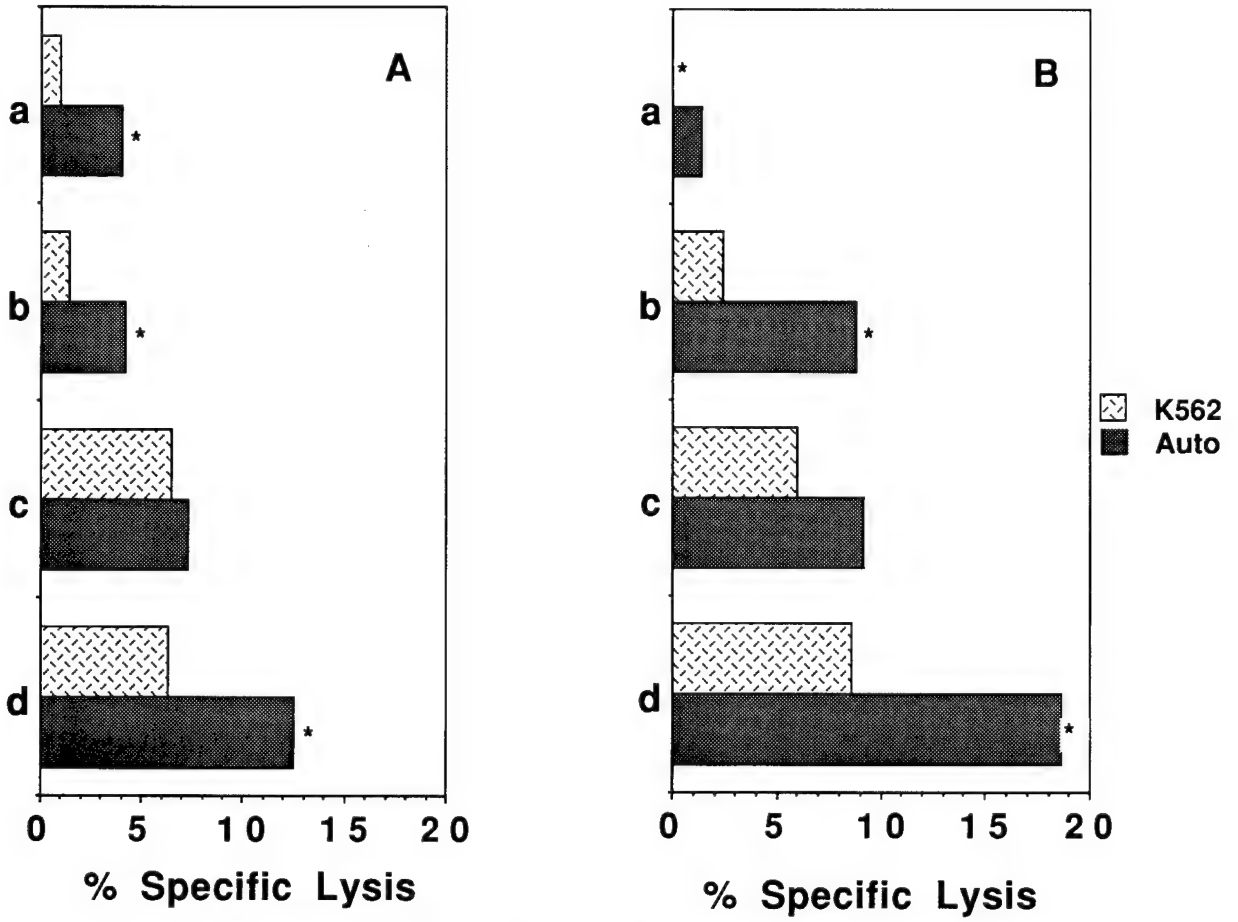

FIG. 3. Target recognition by $CD8^+$ T cells of TAL-1, stimulated in culture with OKT3 mAb. (a) Control TAL-1 bulk culture, no stimulation. (b) TAL-1 bulk culture, stimulated with OKT3 mAb. (c) Isolated $CD8^+$ cells, unstimulated. (d) Isolated $CD8^+$ cells, stimulated with OKT3 mAb. In (A) 5-h assay and (B) 20-h assay, autologous tumor and K562 cells. E:T ratio was 10:1 *Indicates that differences were statistically significant by Student's *t*-test.

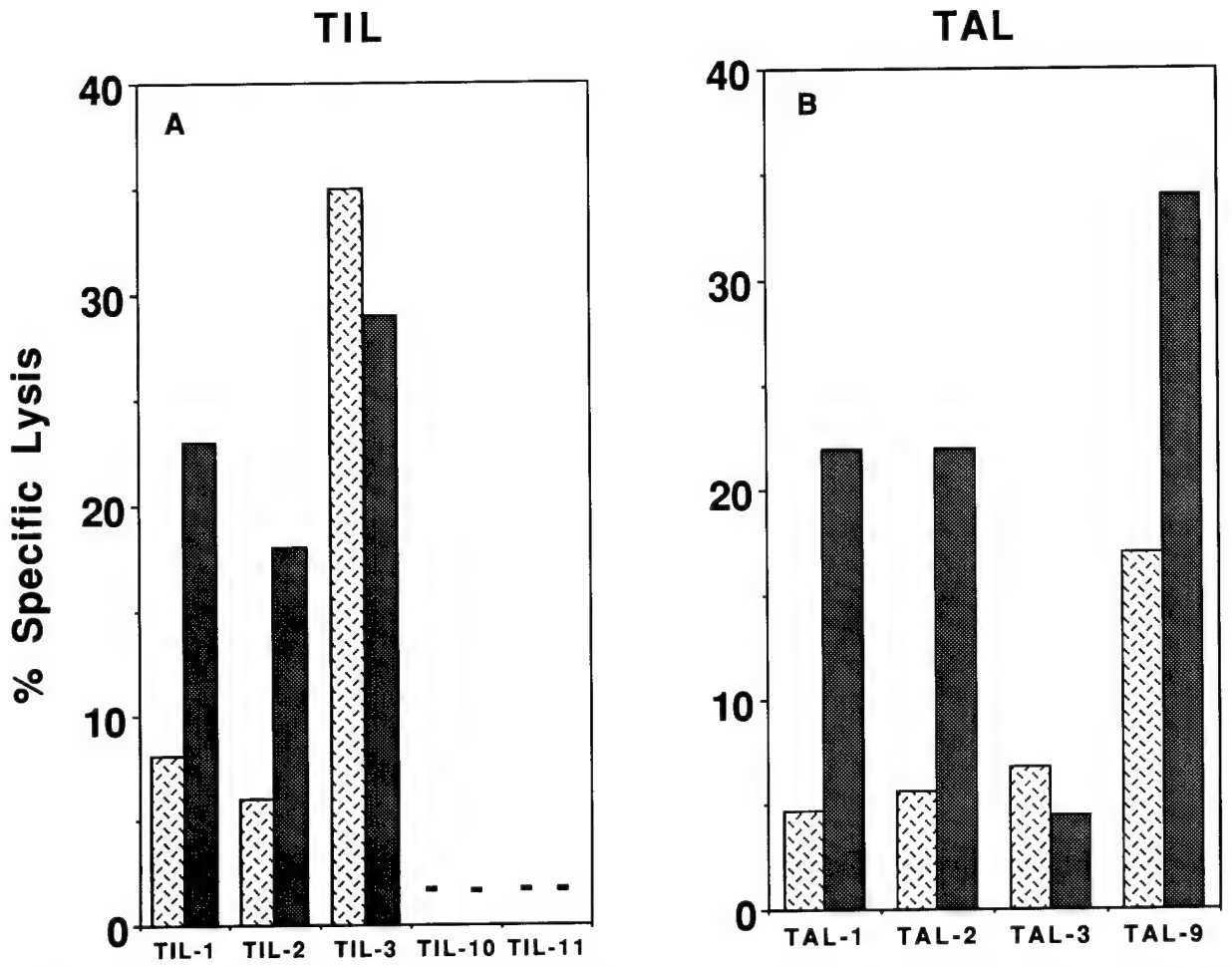

FIG. 4. Recognition by breast TIL and breast TAL of HER-2 peptides. (A) Effectors, breast TIL-10 and TIL-11, isolated from primary tumors were expanded in culture in the presence of IL-2 and initial stimulation with OKT3 mAb. E:T was 20:1. (B) Effectors were TAL-1, TAL-2, TAL-3, and TAL-9 cocultured with autologous tumor only at the initiation of the cultures. The E:T ratio was 10:1. Similar results were obtained at E:T 40:1. One representative experiment of two performed over a 4-week period is shown. Peptides were used in the assay at a concentration of 10 μg/ml. Dash indicates no lysis. (▨) E75, (■) NP indicates that T2 cells were not pulsed with peptides before incubation with effectors.

CTL recognition of ovarian and breast tumors together with that of peptide GP2 (HER-2, 654–662) is also associated with HER-2 overexpression.[22–24] We found that HER-2 peptide E75 (369–377) is also recognized by both breast TIL and TAL. This suggests that multiple HER-2 epitopes may be presented to CTL. This raises the question of whether these CTL epitopes are always present on the tumors. TAL-1 and TAL-2 were characterized by specific recognition of several HER-2 epitopes and low and nonspecific lysis of autologous tumors. TIL-1 and TIL-2 recognized E75, but TAL-10 and TIL-11 did not recognize either E75 or F53 (GP2). TIL-3 and TAL-3 failed to recognize E75, regardless of preferential autologous tumor killing and HER-2 overexpression on autologous tumor cells. This raises several questions. (1) Do these breast CTL recognize Ag that are not present on the autologous tumors at the time of tumor resection but at an earlier stage? (2) Are the antigens present on the tumor but is their density significantly below the density required to sensitize a CTL lytic response? The T2 reconstitution assay has the advantage of amplifying the Ag density by several logs. (3) Is tumor recognition impaired by blocking molecules? The Ag may be present on the tumor, but they cannot induce a CTL response because breast tumors lack costimulatory molecules (e.g., of the B7 family). The lack of IL-2 expression in primary breast TIL has been reported recently.[4]

Conversely, if costimulatory molecules other than the B7 family are present on the tumor and can strengthen the Ag stimulatory signals, differences in Ag processing between professional antigen-presenting cells and tumor cells related to proteolytic machinery precursor stability and availability may lead to presentation of different stimulatory peptides from the same protein during tumor progression. Ongoing studies in our laboratory aim to address these points.

Identification of peptides recognized among the pool of candidate epitopes may allow development of vaccines that can be used to amplify a CTL response to breast cancer earlier, when the tumor is small. Conversely, identification of specificities amplified after repeated stimulation of TIL with autologous tumor (when available) may be useful for adoptive therapies with CTL-TIL plus cytokines. These therapies may be more suitable for patients with advanced disease, who are less likely to respond to cancer vaccination.

ACKNOWLEDGMENTS

The study was supported by Grant DAMD-17-94-J-4313 (C.G.I.) and Nellie B. Connolly Foundation for Breast Cancer Research (J.L.M.).

Peptide synthesis was supported in part by the core grant CA16672. We thank Mr. Bruce Swearingen for expert editorial assistance and Mr. Ben Babcock for technical assistance. We also thank the staff of the Gynecologic Oncology Clinic and Frozen Sections for collecting tumor specimens.

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Received 2 March 1998/Accepted 30 March 1998

Annals of Surgical Oncology, 5(8):743–750
Published by Lippincott Williams & Wilkins

Ovarian Cancer-Associated Lymphocyte Recognition of Folate Binding Protein Peptides

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Background: Tumor-associated lymphocytes (TAL) isolated from ovarian cancer patients contain cytotoxic T lymphocytes (CTL) capable of recognizing specific HLA/peptide complexes on tumor cells leading to tumor cell lysis. Currently, HER2/neu, overexpressed in only 30% of breast and ovarian cancers, is the only known source of CTL-recognized peptides in epithelial cancers. Therefore, we have investigated peptides derived from folate binding protein (FBP), which is over-expressed in more than 90% of ovarian cancers and in the majority of other epithelial tumors.

Methods: TAL were isolated from the malignant ascites of four consecutive HLA-A2$^+$ ovarian cancer patients and incubated in IL-2. Initial chromium-release assays were performed within 1 week. T2 cells, incubated with peptide, were used to reconstitute T cell epitopes. The FBP sequence was interrogated for HLA-A2 binding peptides, and five were synthesized (E37–41).

Results: Freshly cultured, unstimulated ovarian TAL recognize peptides derived from FBP. These peptides are presented in the context of HLA-A2, and are specifically recognized in a HLA class I–restricted fashion. TAL recognition of these reconstituted T cell epitopes is concentration dependent. Furthermore, the FBP peptides are shown by cold target inhibition studies to be naturally processed and presented antigens.

Conclusions: FBP peptides are recognized by freshly isolated TAL from ovarian cancer patients, suggesting in vivo expression and sensitization. Because FBP is over-expressed 20-fold in most adenocarcinomas, these peptides may be used in a widely applicable peptide-based vaccine for epithelial tumors.

Key Words: CTL—Peptide—Vaccine—Folate binding protein

A specific anticancer immune response has been well established in melanoma and, to a lesser degree, in epithelial tumors. However, in cancers of the ovary, breast, lung, and pancreas, tumor-specific cytotoxic T lymphocytes (CTL) have been isolated from tumors, suggesting a host response.[1–4] The most intensely studied epithelial tumor is ovarian cancer.[5–9] This tumor has provided an invaluable model for the study of the specifics and similarities of the immune response to epithelial cancers as compared to melanoma. Ovarian cancer, which is the fourth leading cause of cancer death among American women,[10] offers some unique advantages as a tumor model for immunologic research. This disease often presents in advanced stages with bulky disease and malignant ascites, and the primary treatment involves a staging laparotomy with tumor debulking, resulting in large volumes of solid and ascitic tumor for laboratory use. Tumor-reactive CTL are readily and reproducibly isolated from both the solid tumors and ascites, and the latter can be recollected multiple times without surgery.[1,5–9] The motivation for developing immunologic alternative therapies for this disease is high, because no effective treatments currently exist for women who fail primary platinum or taxol-based chemotherapy.[10]

The classic interaction between the T cell receptor (TCR) on the CTL and the HLA/peptide complex on the tumor cell has been verified in the ovarian model, and as in melanoma, HLA-A2, which is expressed in 50% of Caucasians, has been confirmed as a restriction ele-

Received March 28, 1998; accepted July 12, 1998.

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Presented at the 51st Annual Cancer Symposium of The Society of Surgical Oncology, San Diego, California, March 26–29, 1998.

ment.[11] However, the most promising aspect of ovarian cancer research to date has been the discovery that common CTL-recognized, tumor-associated antigens (TAA) are expressed not only on various ovarian cancers, but also on multiple other epithelial tumors. For example, we have shown that ovarian cancer-specific CTL also recognize common determinants on HLA-matched colon and pancreas cancers.[12] Likewise, in separate studies, we have found that tumor-specific CTL isolated from HLA-matched ovarian and breast cancer patients are cross-reactive for tumor recognition.[13]

In searching for these common TAA, we also have demonstrated the concept of shared TAA among various epithelial tumors by acid elution studies. Using an ovarian cancer cell line[14] and freshly isolated tumor cells,[15] we have eluted the peptide antigens out of the HLA molecules, fractionated them, and reconstituted these epitopes on the HLA-$A2^+$ antigen processing defective mutant T2.[16] We have shown that ovarian, breast, and non–small cell lung cancer-specific CTL recognize the same antigenic fractions, further proving that common TAA exists among epithelial-derived tumors.[14]

The identification of these TAA has progressed slowly despite the rapid developments in vaccine research in melanoma; however, one such antigen system has been found and confirmed by different groups. HER2/neu is a proto-oncogene, and its protein product has been shown to be the source of multiple peptides that are recognized by ovarian, breast, pancreas, and lung cancer-specific CTL.[3,4,13,17,18] Fortunately, HER2/neu expression has been demonstrated in multiple epithelial-derived tumors.[19,20] However, because this is a normal, nonmutated protein, its usefulness as an immunologic target depends on its level of overexpression, and HER2/neu is overexpressed in only 30% of ovarian and breast cancers.[21]

Folate binding protein (FBP), also known as LK26 trophoblast antigen[22] and GP38,[23] is a membrane-associated glycoprotein recognized by the monoclonal antibodies (mAb) LK26, MOv18, and MOv19 and found to be overexpressed in the vast majority of ovarian cancers.[22,24] The level of expression has been found to be more than 20-fold higher in malignant cells than in normal cells,[25] and in one study was reported to be 80- to 90-fold higher.[24] FBP has been the focus of many studies using mAbs,[26,27] folate conjugates,[28,29] and antifolates,[30] but has not yet been investigated as a potential source of CTL-recognized peptides for use in anti-cancer vaccines.

In this study, we demonstrate that fresh tumor-associated lymphocytes (TAL) from consecutive ovarian cancer patients recognize FBP peptides in an HLA-A2–restricted fashion, and that these peptides are naturally processed antigens.

MATERIALS AND METHODS

Tumor-Associated Lymphocyte Cultures

TAL were isolated from fresh collections of malignant ascites obtained through the Department of Gynecologic Oncology at The University of Texas M. D. Anderson Cancer Center under the approval of the Institutional Review Board. Specimens were processed as previously described.[5] Briefly, malignant ascites was collected sterilely in heparinized containers and immediately transported to the laboratory. The cellular elements of the ascites were obtained by centrifugation and washed with serum-free RPMI-1640. Once resuspended, the lymphocytes and tumor cells were separated by centrifugation over discontinuous 75%/100% Ficoll-Histopaque (Sigma, St. Louis, MO) gradients. Freshly isolated TAL were suspended in RPMI-1640 containing 100 μg/mL L-glutamine (Gibco, Grand Island, NY) supplemented with 10% FCS (Sigma), 40 μg/mL gentamicin, and 50 to 100 IU/mL IL-2 (Cetus, Emeryville, CA). T cells were cultured at 0.5 to 1.0 × 10^6 cells/mL and placed in a humidified incubator at 37°C in 5% CO_2 and maintained at this concentration with the addition of media as needed and IL-2 every 2 to 3 days, depending on the growth kinetics. Consecutive specimens were processed and cultured.

Tumor Targets

The SKOv3 ovarian cancer cell line (ATCC, Rockville, MD) was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of HLA-A2 expression, as previously described.[31] This cell line is maintained in RPMI-1640 with 10% FCS and 250 μg/mL G418 (Sigma). Fresh-frozen tumor was collected from the malignant ascites after Ficoll separation and frozen in aliquots in liquid nitrogen until used.

Phenotype Analysis

The HLA-A2 status of these TAL lines was determined by indirect staining with anti–HLA-A2 mAbs BB7.2 and MA2.1 (ATCC), 50 μL of 1 : 50 dilution of culture supernatant at 4°C for 30 minutes, followed by a 30-minute incubation with goat antimouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C Cytometer (Coulter Electronics, Hileah, FL).

Synthetic Peptides

Peptides were synthesized in the Synthetic Antigen Laboratory of The University of Texas M. D. Anderson Cancer Center using solid phase techniques on an Applied Biosystems 430 peptide synthesizer (Applied Biosystems, Foster City, CA). Crude products were dis-

solved and injected onto C-18 4.6-mm I.D. reverse phase HPLC columns (Rainin) and eluted with linear TFA-acetonitrile gradients. Identity and purity of final materials were established by amino acid analysis and analytical RP-HPLC. All peptides used in this study were between 92% and 95% pure. All peptides were derived from the FBP sequence and contained I/L/V at the dominant anchor sites, P2 and P9, necessary for HLA-A2 binding.

HLA-A2 Stabilization Assays

Indirect assessment of peptide binding was performed by HLA-A2 stabilization assays, as previously described.[32] Briefly, T2 cells were pulsed overnight with saturating quantities (100 μg/mL) of each peptide. The cells were then washed and FACS analysis performed as above with BB7.2 and confirmed with MA2.1. HLA-A2 expression was then quantitated as mean channel fluorescence and compared to the expression level on non–peptide-loaded T2. Stabilization is expressed as a ratio of the HLA-A2 expression of peptide-loaded T2 to unloaded T2.

Cytotoxicity Assays

Cytotoxicity was determined by standard chromium release assays, as previously described.[5] Briefly, targets were labeled with 100 to 150 μCi of sodium chromate (Amersham, Arlington Heights, IL) for 1.5 hours at 37°C, then washed twice and plated at 2000 to 2500 cells/well in 100 μL in 96-well round-bottom plates (Costar, Cambridge, MA). Effectors were added at designated effector : target (E : T) ratios in 100 μL per well. After 5 to 20 hours of incubation, 100 μL of culture supernatant was collected, and radionuclide release was measured on a gamma counter (Gamma 5500B, Beckman, Fullerton, CA). All determinants were done in triplicate. Results are expressed as percent-specific lysis as determined by the following formula:

$$(\text{experimental mean cpm} - \text{spontaneous mean cpm}) / (\text{maximum mean cpm} - \text{spontaneous mean cpm}) \times 100.$$

Peptide-Pulsed Cytotoxicity Assays

For these experiments, the T2 cell line (generously donated by P. Creswell) was used. This cell line is a human T cell/B cell fusion product containing an antigen-processing defect in the TAP proteins such that HLA-A2 molecules are empty on the cell surface or contain relatively few bound peptides that can be effectively displaced by exogenous HLA-A2–binding peptides.[16] The T2 cells were labeled with chromium as above, washed, and then incubated with peptide for 1.5 hours at 37°C before standard cytotoxicity assays were performed.

mAb-Blocking Assays

Before the standard cytotoxicity assays were performed, peptide-pulsed T2 was incubated with anti–HLA-A2 mAb BB7.2 (50 μL of 1 : 50 dilution of culture supernatant/well) or anti–HLA-A,B,C mAb W6/32 (ATCC) (5 μL/well) for 30 minutes at 37°C before the effectors were added.

Cold Target Inhibition Assays

Unlabeled T2 was incubated with peptide for 1.5 hours, then added to standard cytotoxicity assays with chromium-labeled tumor targets and effectors. The cold : hot target ratio was 15 : 1. T2 without peptide also was used as a control.

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RESULTS

Folate Binding Protein–Derived Peptides

The FBP sequence was interrogated for potential HLA-A2–binding nonamers utilizing the known binding motifs for this molecule.[8] Five peptides were selected for synthesis, based on the presence of leucine, isoleucine, or valine in the dominant anchor positions P2 and P9, and the potential of these peptides to form amphiphilic structures.[8] Peptides were selected with a wide range of predicted binding affinity. An indirect analysis of HLA-A2 binding was performed with HLA-A2 stabilization assays, which are based on the concept that peptide affinity is directly proportional to enhanced HLA-A2 expression because peptides stabilize the HLA molecules on the cell surface of T2. Therefore, a peptide with high affinity causes more HLA stabilization and higher expression. The peptide sequences, their positions, and their relative binding affinities are listed in Table 1. The HLA-A2 expression is given as a ratio of peptide-induced expression over expression on unloaded T2. Four of the peptides are relatively low-affinity binders, whereas E38 is a high-affinity binder.

FBP Peptide Recognition by Ovarian Cancer–Associated Lymphocytes

Four consecutive ovarian malignant ascites specimens, which were subsequently found to be HLA-A2 positive, were processed, and the TAL isolated and cultured in

TABLE 1. *FBP-derived peptide sequences and HLA-A2 stabilization assays to confirm peptide binding and relative affinities*

FBP peptide	Sequence	HLA-A2 (MCF ratio)*
E37 (25–33)	RIAWARTEL	1.16
E38 (112–120)	NLGPWIQQV	3.64
E39 (191–199)	EIWTHSTKV	1.33
E40 (247–255)	SLALMLLWL	1.18
E41 (245–253)	LLSLALMLL	1.20
No peptide		1.00

* MCF, mean channel fluorescence of FACS analysis with anti-HLA-2 mAb BB7.2. No peptide was utilized as baseline expression of HLA-A2 on T2. Results are expressed as a ratio of the MCF with the specific peptide as compared to no peptide.

IL-2. Standard cytotoxicity assays were performed with the TAL populations within a week of culture initiation to limit in vitro artifact. Figure 1 shows the results of OvTAL1 and OvTAL4 at multiple E : T ratios against all five of the FBP-derived peptides and unloaded T2 as a control. E39-pulsed T2 resulted in the best cytotoxicity with both of these effector populations. The recognition of this peptide by fresh, unmanipulated TAL suggests that these effectors have been previously exposed or primed to this epitope in vivo. To confirm these findings, all four TAL populations were tested against all five peptides in replicated assays performed in triplicate. These results are presented in Figure 2. Several patterns of recognition emerged from these assays, and are indicative of the different T cell repertoires present in the TAL populations. E37, a low-affinity binder, and E38, a high-affinity binder, were not significantly recognized in these assays, and, therefore, served as negative peptide

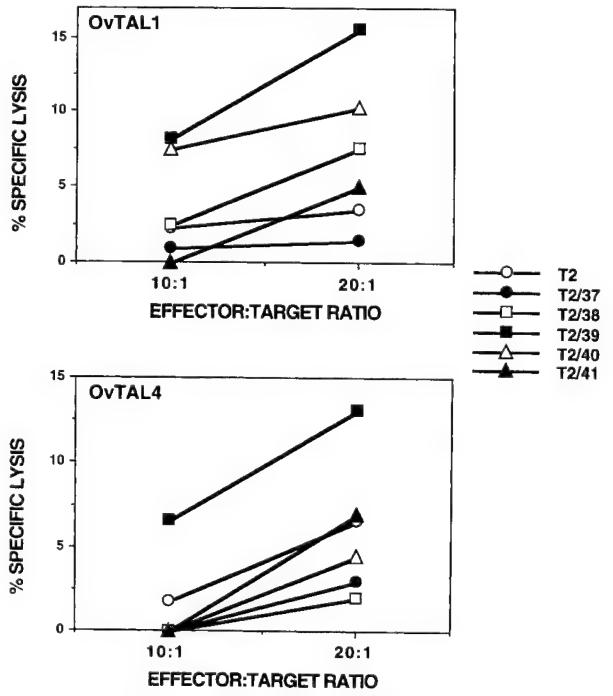

FIG. 1. Freshly cultured ovarian TAL recognize FBP peptides. Consecutive HLA-A2$^+$ ovarian TAL (OvTAL) were isolated from malignant ascites and cultured in IL-2 without specific stimulation. OvTAL1 and OvTAL4 were tested at multiple E : T ratios in standard 5-hour chromium-release assays for recognition of the HLA-A2$^+$ antigen-processing mutant T2,[16] when loaded with five FBP peptides (E37–E41) or no peptide (T2) as a negative control.

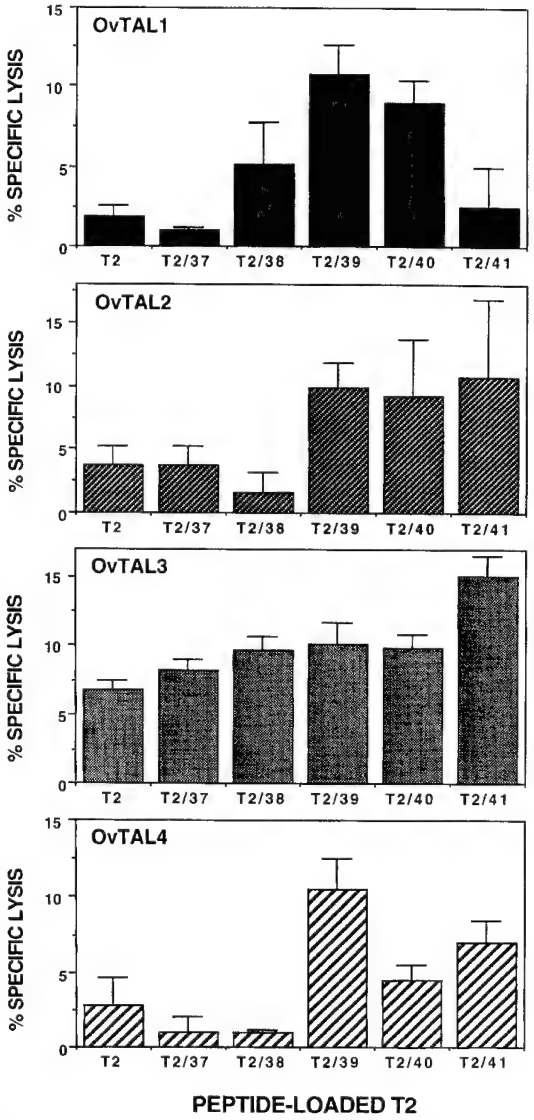

FIG. 2. FBP peptide recognition by consecutive ovarian TAL. Four consecutive HLA-A2$^+$ OvTAL populations were tested against T2 loaded with the five FBP peptides in standard 5-hour cytotoxicity assays. These assays were performed in triplicate at an E : T ratio of 20 : 1 and replicated for each effector. The results are expressed as % specific lysis ± SEM.

controls. E39-loaded T2 was the most consistently recognized target, being lysed by three of four cultures. The lysis data were significant overall when the data was pooled and compared to unloaded T2 or E37 and E38-pulsed T2 ($P < .05$). E39 appears to be the immunodominant FBP-derived peptide, whereas E40 and E41 may serve as subdominant peptides, because each reconstituted T-cell epitopes, with variable recognition.

The Specificity of TAL Recognition of the FBP-derived Peptide E39

To confirm the specific recognition of the HLA-A2/E39 peptide complex on T2 cells by CTL, inhibition assays were performed by initially adding the anti–HLA-A2 mAb BB7.2 to standard cytotoxicity assays. Figure 3*A* demonstrates the successful inhibition of OvTAL2 lysis of T2/39 at multiple E : T ratios by blocking HLA-A2 in 5-hour assays. These data were confirmed with two TAL populations in 20-hour ^{51}Cr-release assays to enhance the sensitivity of the method, as presented in Figure 3*B*. In the latter set of experiments, anti-HLA class I mAb, W6/32, also was utilized to confirm HLA class I presentation of the peptide to CTL because the W6/32 mAb is a more effective blocker of cytotoxicity than is BB7.2. These assays were repeated, and similar results were obtained.

Peptide Concentration-dependent TAL Recognition of FBP-derived Peptide, E39

To better understand the kinetics of CTL recognition of the E39 peptide, T2 cells were split into five parallel cultures and each pulsed with a different concentration of the peptide from 2 to 100 μg/mL for 1.5 hours before standard cytotoxicity assays with the same effector (OvTAL1) at a constant E : T ratio (Fig. 4). The optimal concentration with this low-affinity binding peptide was found to be 50 μg/mL, and half-maximal lysis occurred at the 2 to 5 μg/mL range. This is consistent with the range observed with HER2/neu peptides and most melanoma antigen peptides.[13,18,33] Cytotoxicity dropped considerably at higher concentrations, which has been a consistent finding in other peptide studies.

FBP-derived Peptides Are Naturally Processed Antigens

Cold target inhibition assays were performed to determine whether FBP-derived peptides reconstitute T-cell epitopes that are naturally processed and presented on ovarian tumor cells. Cold T2 were pulsed with E39 and then used to block the cytotoxicity of TAL populations for the ovarian cancer cell line SKOv3.A2. This cell line has been transfected with HLA-A2 in our laboratory and described previously.[31] Figure 5 shows that in multiple

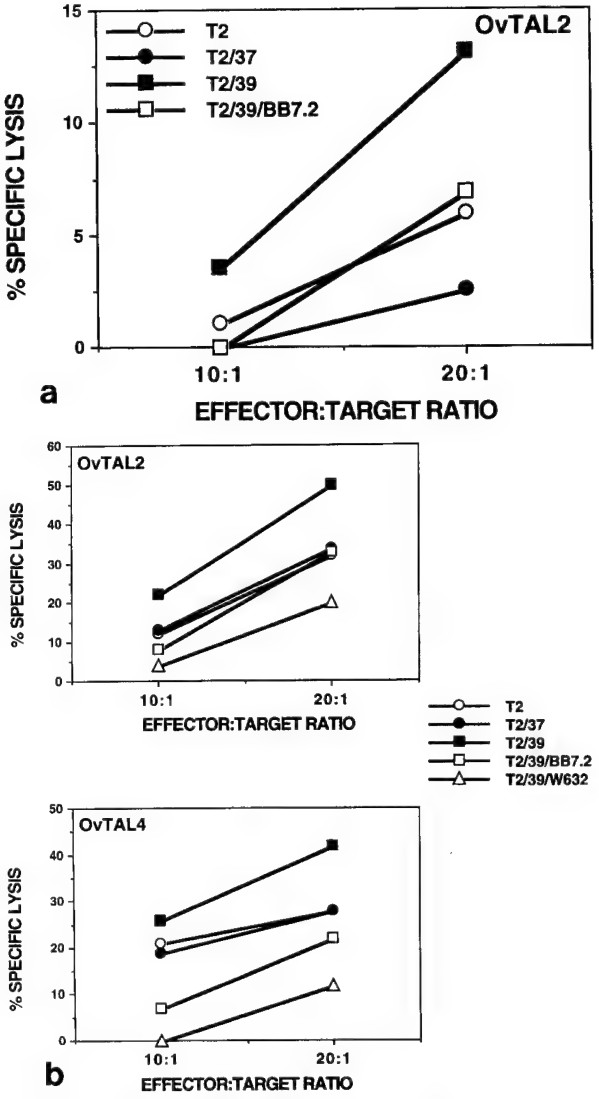

FIG. 3. OvTAL recognition of FBP peptide-loaded T2 is HLA-A2 restricted. mAb blocking assays were performed with the anti–HLA-A2 mAb BB7.2, and the anti-HLA class I mAb W6/32. (**A**) OvTAL2 was tested against T2 loaded with E39, E37, or no peptide in standard 5-hour cytotoxicity assays at multiple E : T ratios. The specific lysis of T2/39 was inhibited by adding BB7.2 to the wells 30 minutes before the assays. (**B**) Both BB7.2 and W6/32 were tested for inhibition of the cytotoxicity of OvTAL2 and OvTAL4 for T2/39 in 20-hour assays at multiple E : T ratios. T2 loaded with no peptide or E37 were used as negative controls. Results are expressed as % specific lysis. Results were confirmed in multiple assays.

assays, T2/39 effectively inhibited 20% to 40% of the tumor lysis by OvTAL1 and OvTAL4 ($P < .05$). These findings suggest that the CTL specific for this epitope contribute significantly to the recognition of this ovarian cancer cell line. Furthermore, these data demonstrate that FBP-derived peptides are naturally processed and presented antigens on intact ovarian tumor cells.

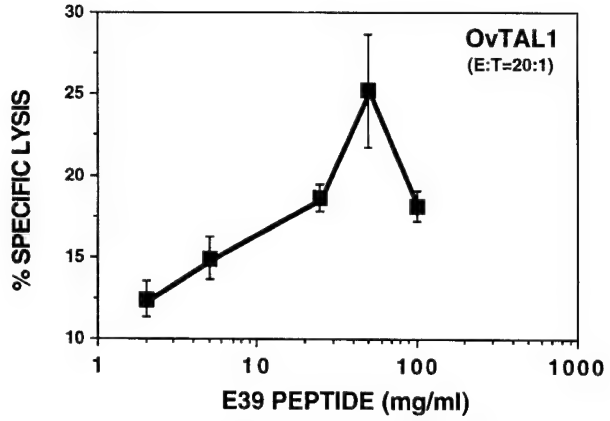

FIG. 4. OvTAL recognition of FBP peptide E39 is concentration dependent. OvTAL1 was tested against T2 incubated with increasing concentrations of peptide in standard 5-hour cytotoxicity assays at an E : T ratio of 20 : 1. The results are expressed as % specific lysis ± SEM.

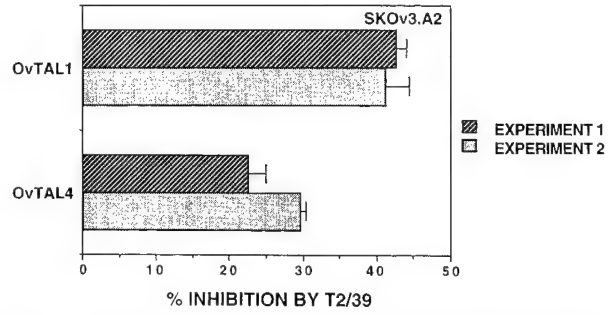

FIG. 5. The FBP peptide E39 is a naturally processed and presented antigen in ovarian cancer. Cold target inhibition assays were performed with OvTAL1 and OvTAL4 in replicated experiments. Cold T2 loaded with E37 (negative control peptide), E39, or no peptide (T2) were tested at a cold : hot ratio of 15 : 1 for inhibition of the recognition of the ovarian cancer cell line SKOv3.A2[27] by OvTAL at E : T ratios of 20 : 1 in 20-hour chromium-release assays. Results are expressed as % inhibition by T2/39 compared to T2/37 and T2.

DISCUSSION

This study demonstrates that FBP is a source of antigenic peptides that induce an endogenous immune response, as shown by the ability of freshly isolated and unmanipulated TAL to recognize several of these peptides, particularly E41 and, most consistently, E39. These peptides were recognized in a HLA-restricted fashion, and the cytotoxicity was concentration dependent. Furthermore, E39 was shown indirectly to be a naturally processed and expressed antigen on the ovarian cancer cell line SKOv3.A2, because peptide-loaded T2 could significantly inhibit CTL killing of this cancer line. Together these data strongly suggest that FBP is an endogenous TAA and is the source of antigenic peptides recognized by TAL in ovarian cancer.

Ovarian cancer has served as an extremely important model for the study of the immune response to epithelial cancer, as demonstrated by the work of multiple groups.[5–9] Most importantly, the findings have been extended to other less studied and technically more challenging epithelial tumor models. Furthermore, many findings in melanoma have been confirmed for epithelial cancers using this model. For example, we now know that an endogenous cellular immune response does exist in a variety of epithelial cancers, and that this response involves the specific recognition of antigenic peptides presented by HLA molecules, specifically HLA-A2, to specific TCR on tumor-infiltrating or tumor-associated CTL.[7,9,11] Unlike melanoma, investigation into the identity of these peptides has so far resulted in only one confirmed TAA, as defined by recognition by cellular immunity. Only the protein product of the oncogene HER2/neu has been shown to serve as a source of endogenously recognized antigenic peptides.[13,18] Unfortunately, this protein is overexpressed in only 30% of all ovarian and breast cancers.[21] The advancement in anticancer vaccine research has been swift in melanoma and has been fueled by the ready supply of multiple commonly expressed TAA.[33] For further development of potentially widely applicable epithelial cancer vaccines, more CTL-recognized TAA must be found for epithelial tumors.

FBP originally was discovered from three independent lines of investigation. The LK26 antigen was identified with a mAb raised against the choriocarcinoma cell line Lu-75(c) by Rettig et al.[34] This antigen initially was found to be expressed in normal as well as malignant trophoblastic cells and eventually in ovarian carcinomas.[22] The MOv18 and MOv19 mAbs were raised against an ovarian carcinoma cell membrane preparation and initially were found to react with a cell surface glycoprotein with a molecular weight of 38 kd.[23] This protein was cloned and sequenced and found to be a high-affinity FBP.[35] Likewise, the latter protein also was characterized from placenta and KB carcinoma cell lines.[36] The LK26 antigen eventually was found to be closely related or identical to the MOv18/MOv19 antigen.[22]

The distribution of FBP expression is extremely interesting and is relevant for immunotherapy. This protein is expressed in some normal specialized epithelium, such as choroid plexus, lung, thyroid, kidney, and sweat glands, but at very low levels.[25] The highest levels of expression of FBP have been found in ovarian carcinomas, and in several independent studies more than 90% of all ovarian carcinomas tested expressed elevated levels of this protein.[22,24] The levels of overexpression have

been shown to be more than 20 times that of normal tissue,[25] and in one study were reported to be as high as 80 to 90 times that of normal tissue.[24] In addition, multiple tumor types have been shown to overexpress the LK26/FBP antigen, including 10 of 11 endometrial cancers, six of 27 colorectal cancers, 11 of 53 breast cancers, six of 18 lung cancers, nine of 18 renal cell cancers, three of three lung carcinoids, and four of four brain metastases from breast cancer. Mesotheliomas, lymphomas, sarcomas, and neuroectodermal tumors were either negative or rarely positive for FBP expression.[22]

The fact that this TAA is so widely and differentially expressed among multiple epithelial tumor types makes it an ideal target for immunotherapy. Multiple attempts have been made to target therapeutic strategies toward FBP, including folate conjugates and antifolates.[28–30] Several studies have involved immunoconjugates or bispecific mAbs, similar to the studies targeting CA-125.[26,27] The use of mAbs against this and other similar cell surface antigens has been limited, because many of these mAbs have been raised against membrane extracts and may have less efficient recognition against the endogenously expressed conformational protein. Also, FBP is shed, and much of the available mAb is bound to circulating antigen. Finally, much of the tumor often is inaccessible to circulating mAbs, and they are rapidly cleared by the host. For these and other reasons, developing mAb-mediated therapies targeting FBP has been a challenge. However, the fact remains that most ovarian carcinomas drastically overexpress FBP, and this antigen is endogenously processed and presented for recognition by cellular immunity.

The FBP-derived peptides shown in this study to be recognized by ovarian cancer-associated CTL may be used in several different immunotherapeutic strategies. First, these peptides could be used to stimulate FBP-specific CTL in vitro for cellular therapy. Adoptive immunotherapy has been shown to reduce tumor burden significantly in up to 30% of end-stage melanoma and renal cell carcinoma patients with TIL.[37,38] In ovarian cancer, TIL in combination with chemotherapy had a synergistic effect, with better results than chemotherapy alone.[39] These results were obtained with uneducated and largely nonspecific TIL, and could be appreciably improved with highly specific CTL directed toward a known TAA. CTL induction studies with FBP peptides currently are underway in our laboratory.

These peptides also may form the basis of a peptide vaccine. Delivery systems for peptide antigens, including dendritic cells and viral vectors, currently are being investigated in melanoma with several ongoing studies.[40] Encouraging results have been reported with the efficient induction of cellular responses in vivo to melanoma-derived peptide antigens effectively delivered.[41,42] We, too, have been investigating HER2/neu-derived peptides in similar strategies and have found efficient CTL induction in vitro with peptide-pulsed dendritic cells.[43] We currently are studying the E75 peptide in clinical trials and virally delivered peptide in vitro. Similar studies will soon be initiated with FBP-derived peptides.

The successful development of a vaccine against epithelial cancer rests on the identification of widely expressed, CTL-recognized antigens that are either exclusively or highly associated with cancerous cells. FBP appears to be the second such known antigen and may be superior to HER2/neu, given its distribution and level of expression as a target for cellular immunity.

Acknowledgments: This work was supported by grants, DAMD17-94-J4313/17-97-17098 (CGI).

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Identification of an Immunodominant Peptide of HER-2/neu Protooncogene Recognized by Ovarian Tumor-specific Cytotoxic T Lymphocyte Lines

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Summary

Synthetic peptide analogues of sequences in the HER-2 protooncogene (HER-2) were selected based on the presence of HLA-A2.1 anchor motifs to identify the epitopes on HER-2 recognized by ovarian tumor–reactive CTL. 19 synthetic peptides were evaluated for recognition by four HLA-A2$^+$ ovarian-specific cytotoxic T lymphocyte (CTL) lines obtained from leukocytes associated with ovarian tumors. The nonapeptide E75 (HER-2, 369-377:KIFGSLAFL) was efficient in sensitizing T2 cells for lysis by all four CTL lines. This peptide was specifically recognized by cloned CD8$^+$ CTL isolated from one of the ovarian-specific CTL lines. E75-pulsed T2 cells inhibited lysis by the same CTL clone of both an HLA-A2$^+$ HER-2^{high} ovarian tumor and a HER-2^{high} cloned ovarian tumor line transfected with HLA-A2, suggesting that this or a structurally similar epitope may be specifically recognized by these CTL on ovarian tumors. Several other HER-2 peptides were recognized preferentially by one or two CTL lines, suggesting that both common and private HER-2 epitopes may be immunogenic in patients with ovarian tumors. Since HER-2 is a self-antigen, these peptides may be useful for understanding mechanisms of tumor recognition by T cells, immunological tolerance to tumor, and structural characterization of tumor antigens.

The existence of CTLs in the leukocyte infiltrations of ovarian tumors, that when expanded in culture in the presence of IL-2 are capable of recognizing autologous, and HLA-matched allogeneic tumors provides strong support to the hypotheses that these CTL recognize multiple private and/or common Ag on tumor and that these Ag can induce T cell responses (1, 2). A critical step towards testing this model is the identification of tumor-specific T cell epitopes. This goal is highly significant because it may lead to an understanding of the immune responses to tumors, the reasons for failure of such responses to control tumor growth in vivo, and development of novel strategies for cancer therapy. Processing of tumor cellular proteins may result in CTL epitopes. In general, the ability of peptide ligand to compete for receptor binding improves as its concentration increases, and the distinction between tumor and normal tissue reactivity may be predicated on the ability of peptides from an overexpressed protein to occupy a significant number of MHC molecules in competition with other peptides according to the laws of mass action (3). Based on these considerations, we proposed the HER-2/neu protooncogene (HER-2)[1] as a potential target for a T cell response against epithelial tumors such as those in breast and ovary, because in a number of tumors the concentration of this protein is increased by up to 100–200-fold over normal tissues. Processing of this overexpressed protein may result in increased peptide supply, which may activate/reactivate an immune response against tumor (3). In support of this hypothesis, evidence from a large case analysis in breast cancer indicates that HER-2 overexpression correlates with a favorable prognosis in patients with breast cancer having a high density of local lymphocyte infiltration (4).

The importance of HER-2 in the recognition of ovarian and breast tumors by CTL in vitro and in vivo has not yet been elucidated, nor have the common epitopes of HER-2 recognized by CD8$^+$ CTL lines from different donors and cloned CD3$^+$CD8$^+$CD4$^-$ CTL been identified. In this study, we have identified common immunogenic epitopes of HER-2 recognized by four out of four and two out of four CD3$^+$CD4$^-$CD8$^+$ ovarian-specific CTL lines that were isolated from tumor-associated lymphocytes (TAL) from HLA-A2$^+$ ovarian cancer patients. CTL clones isolated from one of these lines confirmed recognition of one common HER-2 epitope, and they suggest that a peptide with an identical or cross-reactive sequence is recognized by tumor-reactive CTL on ovarian tumors. Identification of common antigenic CTL epitopes of HER-2 may help to develop targeted immunother-

[1] *Abbreviations used in this paper:* FBP, folate-binding protein; HER-2, HER-2/neu protooncogene; MCF, mean channel fluorescence number; TAL, tumor-associated lymphocytes; TAP, peptide transporter–associated proteins; (TAP1 and TAP2); TIL, tumor-infiltrating lymphocytes.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/95/06/2109/09 $2.00
Volume 181 June 1995 2109–2117

apeutic strategies for breast and ovarian cancer and to elucidate the mechanisms of tolerance towards these epitopes.

Materials and Methods

Synthetic Peptides and Monoclonal Antibodies. HER-2 and control peptides were synthesized by the Synthetic Antigen Laboratory at the M.D. Anderson Cancer Center using a solid-phase method and purified by HPLC. Identity of the final peptides was established by amino acid analysis. The purity of the peptides used in these experiments was ≥97%. mAb to CD3 (OKT3-FITC), CD4 (OKT4-FITC), and CD8 (OKT8-FITC) were obtained from Ortho Diagnostic (Raritan, NJ), mAb W6/32(anti-HLA,-A,-B, -C) from Dako (Dakopatts, Denmark), and anti-HLA-A2 mAb BB7.2 (anti-α-2 domain) and MA2.1 (anti-α-1 domain) from American Tissue Culture Collection (ATCC, Rockville, MD). mAb *Ab2* against HER-2 was obtained from Oncogene Science (Manhasset, NY).

Target Cells and Cell Lines. The human cell line 174CEM.T2 (T2) was a kind gift from Dr. Peter Creswell (Yale University, New Haven, CT). These cells are defective in the normal antigen processing pathway and they express HLA-A2.1 occupied only by signal peptides (5). C1R transfectants C1R:A2 and C1R:A1 cells express HLA-A2.1 and HLA-A1, respectively. Parental Hmy2.C1R (class I reduced) cell line does not express any HLA-A, but expresses low HLA-B35. These cells were a generous gift from Dr. William E. Biddison (National Institute of Neurological Disorders, Bethesda, MD). Tumor lines and leukocytes of the donors of ovarian malignant ascites were phenotyped for HLA-A, -B, and -C antigens by the Blood Bank at the M.D. Anderson Cancer Center. Expression of HLA-A2 on freshly isolated ovarian tumors and transfectants was confirmed by immunofluorescence using MA2.1 mAb. Ovarian tumor lines of known HLA phenotypes, MDAH 2774 (HLA-A3, 24,B45,w57) and SKOV3(HLA-A3,28,B18(w6),35(w6), Cw5), were also used as targets in these experiments.

SKOV3 cells were transfected with the HLA-A2 expression vector RSV.5-neo containing the same full-length HLA-A2.1 cDNA expressed in C1R:A2 cells (6) (kindly provided by Dr. W. Biddison), using the Lipofectin reagent and procedure as described by the manufacturer (Gibco Life Technologies, Grand Island, NY). After selection with G418, clones that expressed high levels of HLA-A2 and HER-2 (as determined by immunofluorescence with MA2.1 and *Ab2* mAbsA) were selected for further experiments.

HER-2 Peptide Binding to HLA-A2.1 To establish the ability of HER-2 peptides to stabilize HLA-A2 expression, the T2 MHC class I peptide stabilization assay was performed as described (7). T2 cells were incubated overnight with saturating amounts of all of the selected HER-2 peptides, as well as with positive and negative control peptides at the same concentration (50 μg/ml). Cells were then washed, stained with BB7.2 and W6/32 mAbs, and analyzed by flow cytometry as described (2, 7, 8). Fluorescence intensity and positions of the peaks were determined using an Epics® V profile analyzer with a log amplifier (Coulter Electronics, Hialeah, FL). Results are expressed as the mean channel fluorescence number (MCF) on a logarithmic scale corresponding to the peak of fluorescence for HLA-A2 (8).

Generation of Ovarian-specific CTL Lines and Clones. CTL were generated by culturing freshly isolated tumor-associated lymphocytes (TAL) from ovarian malignant ascites in complete RPMI medium in the initial presence of autologous ovarian tumor, 25–50 U/ml of IL-2 (Cetus Corp., Emeryville, CA), and 250 U/ml of TNF-α (Genentech, South San Francisco, CA), for 2 wk, followed by selection of $CD8^+$ cells on anti-CD8 mAb–coated culture flasks (AIS Micro CELLector™; Applied Immune Sciences, Menlo Park, CA) and negative selection on anti-CD4 mAb coated flasks as described (9). Isolated $CD8^+CD4^-$ cell lines designated CTLs 1–4 were propagated in culture in complete RPMI medium supplemented with IL-2. $CD3^+CD8^+CD4^-$ clones were established by limiting dilution from CTL-3 line as we described (1, 2).

Identification of Antigenic Peptides. To identify the antigenic HER-2 peptides, CTL lysis of T2 cells preincubated for 60 min with each peptide was measured in a ^{51}Cr release cytotoxicity assay (1, 7). For titration of HER-2 peptides for recognition by $CD8^+$ CTL, T2 cells were incubated with varied concentrations of purified HER-2 peptides. For antibody inhibition experiments, targets were preincubated with the appropriate antibody as described (2), then washed and incubated with effectors. Percentage of specific lysis was determined from the equation $(A - B)/(C - B) \times 100$, where A is lysis of T2 cells by effectors in the presence of a peptide, B is spontaneous release from T2 cells in the presence of the same peptide but in the absence of effectors, and C is the maximum ^{51}Cr release. The experiments were performed in triplicate, and the mean ± SD values were calculated from at least two separate experiments. Since even one amino acid change in peptide length at the COOH-terminal end can have dramatic effects on peptide recognition (10), and identification of CTL epitopes is performed with synthetic peptides, cytotoxicity values were considered to indicate significant recognition of a peptide when the differences between mean ± SD values for percent of specific lysis of T2 cells preincubated with a peptide or medium were ≥10%, at an E/T ratio of 20:1 (10) and statistically significant ($p < 0.05$). Cold target inhibition of cytolysis was performed using ^{51}Cr-labeled ovarian tumors, OVA-1 (autologous with CTL-1), as well as SKOV3.A2.1E4 transfectants as "hot" targets and T2 cells pulsed with peptides as "cold" targets. T2 cells were preincubated with HER-2 or control peptides (50 μg/ml) overnight, then washed and admixed with ^{51}Cr-labeled targets at 5:1 and 10:1 (cold/hot) target ratios.

Statistical Analysis. Values obtained for percent of specific lysis by the same effectors of T2 cells preincubated with HER-2 peptides and percentage of specific lysis of T2 cells in the absence of exogenous peptides were examined by the Student's t test. Differences were considered significant when the $p < 0.05$.

Results

Identification of HER-2 Peptides That Stabilize HLa-A2.1 Expression. To identify the HER-2 epitopes recognized by these CTL, 19 peptides were selected from the HER-2 sequence based on the HLA-A2 anchor motifs based on the presence of Leu/Ile at position 2 (P2) and Val/Leu/Met at P9 (11, 12). The majority (16 out of 19) peptides selected for this study were nonamers. Two octamers were included because they were part of overlapping epitopes. 15 out of 19 peptides contained Leu (P2), while two peptides contained Ile (P2) and two peptides contained Val (P2). Only octa and decamers were found in the HER-2 sequence to contain Met at P2, and consequently these peptides were not included in the present study. Peptides were selected from signal, extracellular, transmembrane, and cytoplasmic domains of HER-2 (13). Priority was given to peptides from the regions 364-474 and 781-859 because they contain the highest density of continuous and overlapping epitopes with HLA-A2.1

binding motifs. Peptides were also selected from the signal and transmembrane domains of HER-2 because hydrophobic, Leu-, Ile-, and Val-rich peptides were found bound on HLA-A2 of both T2 and C1R:A2 cells (5, 14), were described as CTL epitopes (10), and may be bound to HLA-A2 in transporter-associated proteins (TAP)-deficient targets (5). The peptides selected for this study represent more than 50% of all nonamers that are potential HER-2 epitopes with HLA-A2 anchor motifs (11, 12).

Since both P2 and P9 anchors and residues from the central area of peptide contact HLA-A2 and define the affinity of a peptide to the presenting molecule (12), to determine the binding ability of HER-2 peptide analogues, expression of HLA-A2 on T2 cells was determined in the presence of each HER-2 peptide and the corresponding control peptides. All peptides were tested for binding to the MHC class I in the HLA-A2 stabilization assay using T2 as indicator cells (7, 8). To establish that the results reflect MHC class I heavy chain expression indicative of absolute binding and not only the effects of peptide-induced conformational changes (8) that may affect the reactivity of mAb with the peptide-binding pocket, T2 cells were stained with both W6/32 (anti–class I monomorphic) and BB7.2 (anti–HLA-A2 α-2 domain) mAb. As shown in Table 1, 9 out of 19 HER-2 peptides (ranked 1–9 based on their HLA-A2– stabilizing ability) induced a greater than twofold increase in MCF for HLA-A2 expression, compared to negative control peptide C61, as determined with BB7.2 mAb. Similar results were obtained after staining with MA2.1 mAb (data not shown). HLA-A2 stabilization for E75, E90, and E89 was peptide concentration dependent in the range 1–50 μg/ml (not shown). This suggests that these peptides have higher stabilizing ability of both conformational epitope BB7.2, as well as HLA-A2 molecule expression than the other 10 peptides (ranked 10–19), which were designated as peptides with low stabilizing ability for HLA-A2.1. Five other peptides, E70, E71, E72, D97, and D99, did not affect serological epitope W6/32 expression, suggesting that they bound poorly to HLA-A2. Of these peptides, the octamer D97 induced a significant increase in BB.7.2 epitope expression, suggesting induction of a conformational epitope rather than stabilization of HLA-A2 expression. In contrast, BB.7.2 bound poorly to T2 cells pulsed with peptide E74 compared with W6/32. The implication of this serological analysis is that HER-2 peptides, in addition to having binding and stabilizing effects on HLA-A2 expression, may lead to conformational changes in the Ag-binding pocket.

Recognition of HER-2 Peptides by Ovarian Tumor–reactive CTL. To identify HER-2 peptides recognized by ovarian tumor–reactive CTL lines, four $CD8^+$ CTL lines designated CTLs 1–4 were generated from cultured TAL from four different $HLA\text{-}A2^+$ donors after $CD8^+$ cell selection on anti-CD8 antibody–coated plates. These CTL lines were 100% $CD3^+$, 100% $CD8^+$, and 0–2% $CD4^+$. This approach was considered necessary because the Ag specificity of $CD3^+$ $CD8^+CD4^-$ CTL isolated from tumor-infiltrating lymphocytes (TIL)/TAL, which have been in culture for 2 wk, will not be diluted or masked by the overgrowth of $CD4^+$ cells, which is encountered in long-term TIL/TAL cultures. To avoid changes in the Ag specificity, the isolated $CD3^+CD8^+$ $CD4^-$ lines were not restimulated with autologous or allogeneic $HLA\text{-}A2^+$ tumors. These CTL lines recognized autologous and allogeneic $HLA\text{-}A2^+$ ovarian tumors, but not $HLA\text{-}A2^-$ ovarian tumors or lines, as illustrated in Table 2. Since freshly isolated ovarian tumors from different donors may be antigenically heterogeneous or may express variable levels of HER-2, we needed as target a cloned ovarian tumor of high and stable HER-2 protein expression and known HLA-phenotype having HLA-A2 in common with the effectors. Tumor cells of the SKOV3 line, which overexpresses HER-2 protein (15), were transfected with the HLA-A2 gene. TAP1 and TAP2 message expression in SKOV3 is increased in parallel with HLA class I by IFN-γ treatment (16), suggesting unimpaired Ag-presenting ability. A clone SKOV3.A2.1E4 expressing high and stable levels of HER-2 protein and HLA-A2 was used as a target in these experiments. Four $HLA\text{-}A2^+$ ovarian $CD8^+CD4^-$ CTL lines lysed SKOV3.A2.1E4 clone in addition to autologous and allogeneic $HER\text{-}2^{high}$ $HLA\text{-}A2^+$ tumors. They did not recognize $HLA\text{-}A2^+$, $HER\text{-}2^{low}$ ovarian cell lines. Furthermore, they did not lyse K562 cells, indicating that they did not express NK or LAK activity (Table 2). Autologous tumor lysis was inhibited by mAb to CD3 TCR (OKT3) and HLA-A2 (MA.2.1), but not by anti–HLA-DR mAb, suggesting that they can recognize Ag presented by HLA-A2 (data not shown).

To evaluate whether these $CD8^+$ CTL recognized the same or different HER-2 peptides, lysis of T2 cells preincubated with each peptide was tested with all CTL lines. Both high and low affinity peptides were tested in the same experiment since it has been reported that a melanoma CTL epitope is derived from low affinity HLA-A2–binding peptides (17). For increased stringency in epitope identification, recognition of an HER-2 peptide was considered significant based on convergence of results of statistical analysis of differences in cytotoxicity data (18, 19) and assigning a cut-off value of at least 10% for the differences between recognition of T2 cells exogenously loaded with HER-2 peptides and T2 cells presenting only endogenous peptides. This approach was necessary because we wanted to identify peptides that, based on the levels of observed lysis, are either recognized with higher affinity than others or their recognition reflects the presence of a higher percentage of specific reactive clones.

Based on comparison of cytotoxicity values for T2 cells, lysis by $CD8^+$ CTL1-4 in the presence and absence of HER-2 peptides, CTL-1 and CTL-2 recognized mainly peptide E75 (Fig. 1). CTL-3 recognized, in addition to E75, three other peptides (E90, E89, and C85), but it did not recognize the remaining 15 peptides. CTL-4 recognized four of the peptides tested, including E75 and C85. CTL-4 recognized, at a lesser extent than E75 and C85, two other HER-2 peptides residues, 799-807 (E71) and 835-842 (E73). Peptides E71 and E73 were not recognized by the other three CTL lines, even when reconstitution of the epitopes was attempted at either higher peptide concentrations or higher E/T ratios, and may represent private epitopes for CTL-4 (Fig. 1). Since CTL-4

Table 1. *Cell Surface Expression of BB7.2 Epitope on HLA-A2.1 of T2 Cells by HER-2 Peptides*

Code	Position	1	2	3	4	5	6	7	8	9	10	BB7.2 MCF*	BB7.2 Rank‡	W6/32 MCF	W6/32 Rank
HER-2 peptides															
E91	5-13	A	L	C	R	W	G	L	L	L		82	9	306	8
D97	42-49	H	L	D	M	L	R	H	L			52	12	167	18
D113	48-56	H	L	Y	Q	G	C	Q	V	V		155	2	496	2
E75	369-377	K	I	F	G	S	L	A	F	L		131	3	474	4
E77	391-399	P	L	Q	P	E	Q	L	Q	V		61	10	216	11
E76	402-410	T	L	E	E	I	T	G	Y	L		109	6	358	5
E78	457-465	S	L	R	E	L	G	S	G	L		60	11	208	12
E93	466-474	A	L	I	H	H	N	T	H	L		113	5	293	9
E92	650-658	P	L	T	S	I	I	S	A	V		128	4	324	6
E88	689-697	R	L	L	Q	E	T	E	L	V		109	7	481	3
E70	793-801	T	V	Q	L	V	T	Q	L	M		35	18	172	17
E90	789-797	C	L	T	S	T	V	Q	L	V		164	1	515	1
E71	799-807	Q	L	M	P	Y	G	C	L	L		42	16	173	15
E72	828-836	Q	I	A	K	G	M	S	Y	L		32	19	166	19
E73	835-842	Y	L	E	D	V	R	L	V			51	13	234	10
E74	838-846	D	V	R	L	V	H	R	D	L		36	17	203	13
E89	851-859	V	L	V	K	S	P	N	H	V		82	8	310	7
C85	971-979	E	L	V	S	E	F	S	R	M		47	14	194	14
D99	1089-1098	D	L	G	M	G	A	A	K	G	L	44	15	172	16
Control peptides§ HER-2															
C81	971-979	E	L	V	S	E	V	S	K	V		76		261	
C61	968-977	R	F	R	E	L	V	S	E	F	S	37		182	
Folate-binding protein‖															
E38	112-120	N	L	G	P	W	I	Q	Q	V		77		N.D.	
E37	25-33	R	I	A	W	A	R	T	E	L		34		N.D.	
E41	245-253	L	L	S	L	A	L	M	L	L		38		N.D.	
No peptide												34		172	

* Mean channel fluorescence (MCF) corresponding to the peak of fluorescence for T2 cells preincubated with 50 μg/ml of each peptide was determined for all peptides in the same experiment as described in the Materials and Methods. MCF for both W6/32 and BB7.2 are presented and compared to confirm the increase in MHC class I heavy chain expression.

‡ Peptides are ranked in decreasing order of their ability to increase HLA-A2.1 expression.

§ The variant peptide of C85 containing three substituted residues F → V(P6), R → K(P8), and M → V(P9) was used as positive control because the resulting variant (C81) contains four dominant and strong anchor residues (11, 12) reported to favorize binding to HLA-A2. The peptide C61 (HER-2: 968-977) contains HLA-B8 anchors and was used as negative control.

‖ Folate-binding protein (FBP) peptides were selected from the FBP sequence based on the concordance of T cell epitopes predicted by the computer program, ANT.Find.M (3), and the presence of HLA-A2–specific anchor motifs (11).

was isolated from the ascites corresponding to previously reported TAL-24, these results confirm the recognition of a peptide from the area 968-984 (C85) as a potential T cell epitope derived from HER-2 or a structurally similar peptide HLA-A2 complex (20). Peptide E75 was recognized by all four CTL lines, C85 by two out of four CTL lines, while peptides E89 and E90 were recognized by CTL-3, and E71 and E73 by CTL-4. All four CTL lines failed to specifically recognize a number of HER-2 peptides presented by T2 cells with canonical HLA-A2 anchors at P2 and P9 and different central sequences, including D113, which was reported to bind HLA-A2 with high affinity (12). All CTL lines showed low levels of lysis of T2 cells without exogenous peptides (T2 cells present a number of signal peptides) (5), comparable

Table 2. *Recognition of HLA-A2⁺ Tumors by Ovarian-specific CTL Lines*

Targets‡	Percent of specific lysis*			
	CTL-1	CTL-2	CTL-3	CTL-4
Auto-T (HER-2⁺,A2⁺)	47	65	28	41
Allo-T (HER-2⁺,A2⁺)	40	41	14	38
SKOV3.A2.1E4 (HER-2⁺,A2⁺)	45	39	42	84
Allo-T (HER-2⁻,A2⁻)	5	15	NT	3
2774 (HER-2⁻,A2⁻)	0	0	0	4
K562 (HER-2⁻,A2⁻)	1	3	3	4

* Percent specific lysis is shown for an effector to target ratio of 20:1. Target lysis was determined in a 5-h 51Cr release assay. NT, not tested.
‡ Auto-T and Allo-T represent autologous and allogeneic freshly isolated ovarian tumors. SKOV3.A2.1E4 is an ovarian tumor clone expressing HLA-A2. 2774 is a human ovarian tumor line.

to lysis of the NK targets, K562 cells. These results show that ovarian tumor–reactive CTL can recognize common HER-2 epitopes, although the pattern of peptide recognition is different for each line.

Recognition of E75 by CTL3 Clones. Results presented above are suggestive of common HER-2 peptide recognition by four distinct CTL lines. We wanted to establish whether E75 is specifically recognized by cloned CTL, and whether it may correspond to an epitope recognized by the same CTL clone on ovarian tumors that overexpress HER-2. CTL-1 and, to a lesser extent, CTL-2, appear to be highly restricted in their recognition of E75. This can cause the results of peptide specificity experiments with cloned CTL-1 and -2 to appear biased in the favor of E75. To address the question of whether reactivity to E75 was a property of distinct non–cross-reactive clones from a line of multiple HER-2 specificities, and to establish whether these clones recognize E75 in a peptide concentration–depependent and –specific manner, clones were developed from CTL-3 by stringent limiting dilution and further expanded in culture.

CTL-3 line did not lyse the C1R or C1R:A2 cells (Fig. 2 *A*). Since C1R:A2 and SKOV3.A2.1E4 were transfected with the same plasmid carrying the same HLA-A2 gene, this suggests that the endogenous peptides recognized on SKOV-3.A2.1E4 by CTL-3 may be different from the ones presented by C1R:A2 cells. Both E75 and C85 were recognized by CTL-3 and CTL-4 when presented by C1R:A2 but not by C1R:A1 transfectants (data not shown). Concentration-dependent recognition of E75, C85, and E90 was observed with CTL-3 (Fig. 2 *B*). This recognition was confirmed in independently performed experiments. Documentation of restricted expression of FBP on ovarian tumors suggests the possibility of cellular immune recognition of FBP peptides (21). CTL-3 did not recognize two peptide analogues of FBP, residues 112-120 (E38) and residues 245-253 (E41) (Fig. 2 *A*). FBP peptides were selected to include HLA-A2–binding anchors and to exhibit high (E38) and low (E41) HLA-A2– binding affinity, respectively (Table 1). Similarly, CTL-3 did not recognize two peptide analogues of the Muc-1 core peptide (22), D125 and D132. Recognition by cultured ovarian TAL of Muc-1 core exposed on ovarian tumors and of Muc-1 gene transfected and expressed by EBV-B cell lines has been suggested (23). Muc-1 core sequence lacks canonical HLA-A2 anchors at correct distances to allow binding to the main HLA-A2 pockets (21), but its recognition has been described mainly as non-MHC restricted (24). Therefore, in both Muc-1 peptides, GLTSAPDTRV (D125) and SLADPAHGV (D132), HLA-A2 anchors were introduced (underlined) to engage binding and to present the intervening sequence to TCRs. These results, together with the results presented in Fig. 1, suggest that

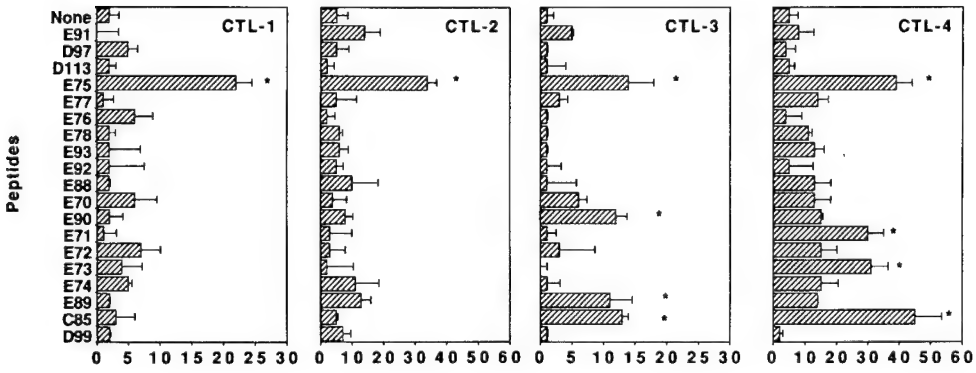

Figure 1. Recognition of HER-2 peptides by CD3⁺CD8⁺CD4⁻ CTL isolated from four different ovarian cancer patients. Cytotoxicity was determined using T2 cells preincubated for 60 min with each peptide at 25 μg/ml in a 5-h ^{51}Cr release assay. Percentage of specific lysis is shown for all CTL lines for an E/T ratio of 20:1. Percentage of specific lysis was calculated as described in Materials and Methods. Asterisk indicates mean cytotoxicity values that are at least 10 percentage points greater than mean values for the lysis of T2 in the absence of peptide and are also significantly different by Student's *t* test ($p < 0.05$).

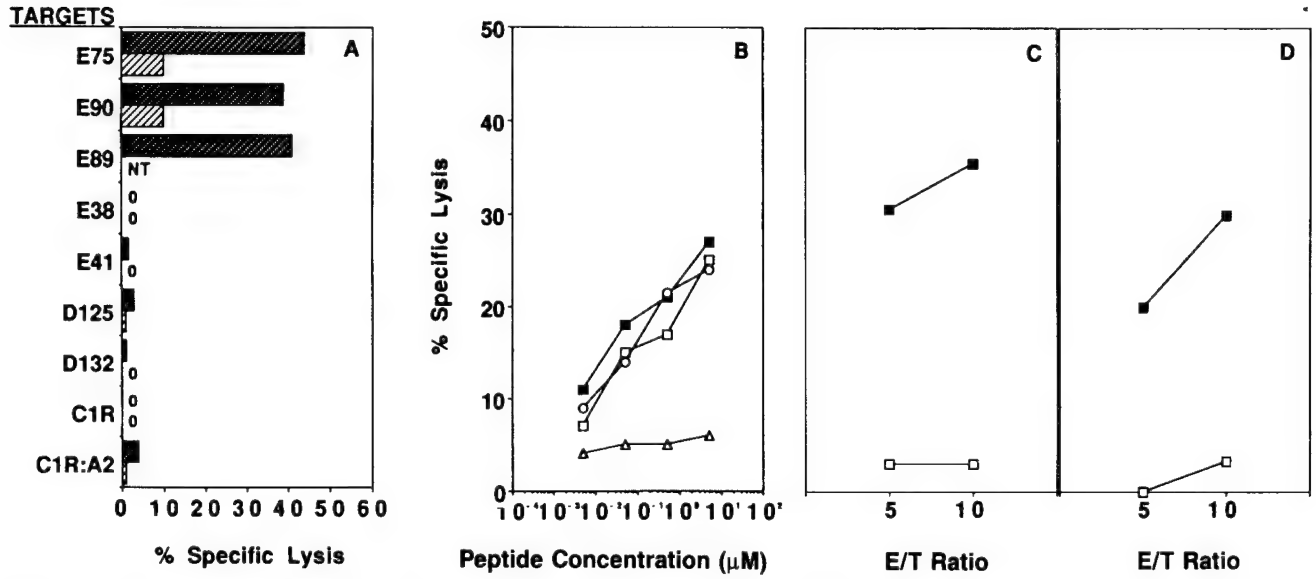

Figure 2. Recognition of E75 by CTL-3 clones. (*A*) Lysis by the CTL-3 line. 3000 ^{51}Cr-labeled T2 cells were incubated with HER-2 peptides E75, E89, and E90, FBP peptides E38 and E41, and variant Muc-1 peptides D125 and D132 at a final concentration of 25 μM for 60 min before effectors were added. Supernatant was collected and counted after 5 h. E/T ratios were 10:1 (▨) and 5:1 (▩). Results are presented as the percentage of specific lysis by effectors of T2 cells pulsed with peptides. The same numbers (3,000) of C1R and C1R:A2 cells were used as targets. (*B*) Concentration-dependent recognition of E75 (■), E90 (□), C85 (○), and E92 (△) by CTL-3 line at an E/T ratio of 20:1. (*C* and *D*) Lysis by clones 3C4F (*C*) and 3B4E (*D*) of E75- (■) and E90- (□) pulsed T2 cells. Lysis of T2 cells incubated with E89 at 25 μM was 7% by clone 3C4F and 5% by clone 3B4E at 10:1 E/T ratios.

CTL-3 line contains clones that are specific for particular peptide epitopes.

Two clones, 3C4F and 3B4E, isolated from CTL-3 line that recognized E75 but not E90 or E89 peptides presented by T2 cells, are shown in Fig. 2 (*C* and *D*). Recognition of T2 cells incubated with the same concentration of E75 suggest that clones 3C4F and 3B4E are specific for peptide E75 (Fig. 2, *C* and *D*). Recognition of E75 by these two clones was compared over a range of concentrations (10 nM–10 μM) (Fig. 3 *A*). At an E/T ratio as low as 4:1, peptide E75 reconstituted T cell recognition by clone 3C4F at a concentration (100 nM) similar to that reported for an HLA-A2–restricted epitope gp100 and an HLA-A1–restricted epitope from MAGE-3 recognized by melanoma-specific CTL (25, 26), but at higher

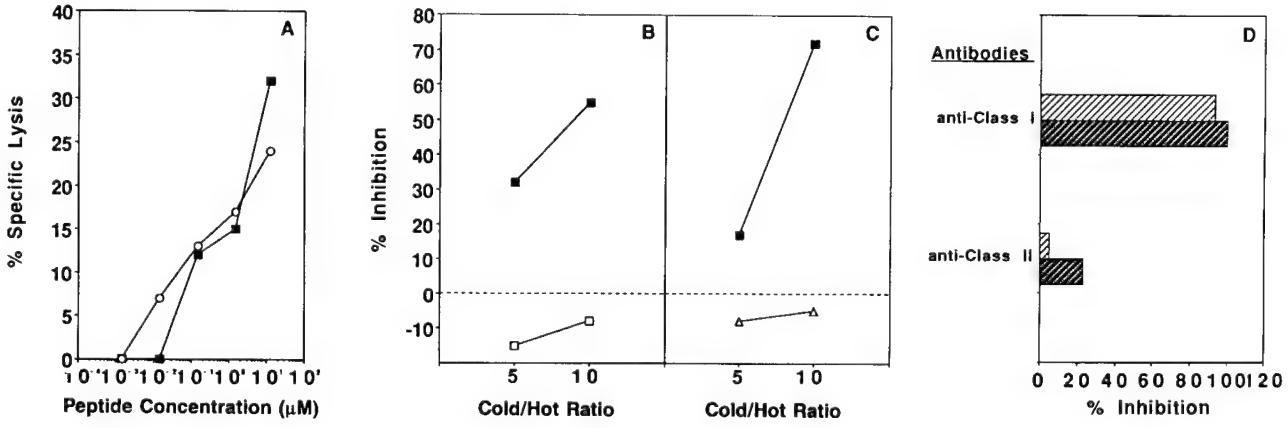

Figure 3. Inhibition of ovarian tumor recognition by clone CTL-3C4F by T cell epitope E75. (*A*) Dose-response recognition of peptide E75 by clones 3C4F (■) and 3B4E (○). Serial dilutions of peptide E75 were incubated with 3,000 T2 cells for 60 min. CTL were added at a E/T ratio of 4:1, and a standard 5-h cytotoxicity assay was performed. Lysis of T2 cells preincubated with E90 at 10 μM was <5% by both CTL clones. (*B* and *C*) Cold-target inhibition of lysis of freshly isolated ovarian tumor OVA-1 (*B*), and ovarian tumor clone SKOV3.A2.1E4 (*C*) by T2 cells preincubated with peptides E75 (■), E90 (□), and D132 (△). The effector (CTL-3C4F clone)/hot target ratio was 10:1. Peptide pulsed T2 cells (cold targets) were added in the assay at 5:1 and 10:1 cold/hot target ratios. Inhibition of lysis was determined in a 5-h ^{51}Cr release assay. Results are presented as percentages of inhibition of tumor target lysis by clone 3C4F, which was 46% for OVA-1 (*B*) and 28% for SKOV3.A2.1E4 (*C*). Lysis of parental control targets SKOV3 (HLA-A2$^-$) was 6% and of D132-pulsed T2 cells was 5% at the same E/T ratio. (*D*) Lysis of SKOV3.A2.1E4 was inhibited by anti–HLA class I (W6/32 mAb) but not by anti–HLA-DR (L243 mAb) at both 10:1 (▨) and 5:1 (▩) E/T ratios.

E/T ratios. These peptide concentrations are, for MAGE-3, gp100 and HER-2, significantly higher by at least two orders of magnitude than those of HLA-A2.1–restricted viral proteins (27).

To confirm that clone 3C4F recognizes a natural epitope associated with HLA-A2 on ovarian tumors, we examined the ability of E75 and E90 pulsed T2 cells to inhibit lysis of freshly isolated OVA-1 because CTL-1, autologous with this tumor, recognize only E75. Significant inhibition of lysis was observed by T2 cells pulsed with E75 but not with E90 (Fig. 3 *B*). Since antigen expression on freshly isolated ovarian tumors can be heterogeneous, to confirm that E75 represents an epitope presented by HLA-A2 on an ovarian tumor clone, the ability of E75 pulsed T2 cells to inhibit lysis of clone SKOV3.A2.1E4 was examined. Both OVA-1 and SKOV3.A2 transfectants share only HLA-A2 with effectors and express HER-2 on the surface, OVA-1: 79% HER-2$^+$ cells, MCF = 29; SKOV3 (positive control): 100% HER-2$^+$ cells MCF = 40; SKOV3.A2.1E4: 100% HER-2$^+$ cells, MCF = 40; C1R:A2 cells (negative control) MCF = 0.7. Ovarian clone SKOV3.A2.1E4 lysis by 3C4F clone was inhibited by anti–MHC class I (W6/32) but not by anti–MHC class II mAb (Fig. 3 *D*). Again, significant inhibition of lysis by clone 3C4F was observed in the presence of E75. Control peptide D132, which was not recognized when pulsed on T2 cells, failed to redirect clone 3C4F lysis (Fig. 3 *C*). Therefore, E75, which is recognized by four CTL lines and cloned CTL isolated from one of these lines, and which specifically inhibits recognition of ovarian tumors, may be a natural common HER-2 epitope recognized by ovarian-specific CTL.

Discussion

In this study, we have investigated recognition of synthetic peptide analogues of HER-2 epitopes containing HLA-A2–binding motifs by CD8$^+$CD4$^-$ CTL lines and clones isolated from TAL with ovarian tumors. We have identified one common epitope (E75) that is dominantly recognized by four out of four CTL lines. Of 19 peptides tested, another common epitope, C85, is recognized by two out of four lines. Several other epitopes, E89, E90, E71, and E73, are recognized only by one of the four CTL lines used, suggesting that they may be either private epitopes for these CTL or clones recognizing these epitopes are present with low frequency in the other CTL lines. The second possibility is more likely because the pattern of concentration-dependent recognition for E90 is similar with that of E75 and C85. In certain experiments, statistical analysis found that recognition of E89 and E90 by CTL-2 and CTL-4, and E91 by CTL-2 was significantly different from control targets, but the levels of recognition were lower (5–7%) than the cut-off value. We have observed that some ovarian CTL cultures lose the ability to recognize a number of these peptides over time probably because of gradual loss of lytic function or overgrowth of CTL of different specificities (Ioannides, C. G., and B. Fisk, unpublished data). We confirmed the specificity of E75 recognition by using two clones isolated from one of the CTL lines. E75 effectively inhibited lysis by CTL clones of both a freshly isolated ovarian tumor and an ovarian tumor line transfected with HLA-A2, indicating that the epitope recognized is not a culture artifact. Control peptides containing HLA-A2 anchor motifs (11, 12) but different intervening sequences failed to inhibit lysis, suggesting that a natural peptide with an identical or cross-reactive sequence is immunogenic in HLA-A2 ovarian cancer patients and may be presented on ovarian tumors.

Both E75 and C85 were recognized with different efficiencies by CTL1-4 at the same peptide concentration. This may be caused by the existence of clones in these CTL lines that recognize other as yet unknown antigens. The existence of multiple distinct ovarian Ag expressed simultaneously on the same tumor clone has been shown by analyzing recognition of ovarian clones by CTL isolated from TAL (1). Ovarian-specific CTL lines restricted by HLA-A2 recognize common epitopes present on allogeneic HLA-A2$^+$ ovarian tumors or lines, but not on HLA-A2$^+$ melanomas. Individual ovarian-specific CTL lines were found to recognize multiple Ag epitopes. Some of the common determinants may be expressed on other HLA-A2$^+$ epithelial tumors (2, 3). We have previously shown that ovarian TAL can recognize Muc-1 core peptides and HER-2, 968-984, a longer analogue of C85 peptide (HER-2, 971-979) (20). CTL-4 was isolated from TAL-24 used in these studies (20, 23). Recognition of Muc-1 by at least some of the clones derived from the CTL lines used in this study is likely. Although the percentage of tumor cells expressing Muc-1 in a tumor sample is variable and its expression is heterogenous, most ovarian tumors (>80% of serous adenocarcinomas) have been reported to express Muc-1 (23). Another HER-2 peptide, 654-662, derived from the transmembrane domain, was found to be recognized by TIL isolated from non–small cell lung cancer and developed by different methods (19). HER-2 is expressed in ~30% of ovarian and breast carcinomas. However, its expression is relatively stable over time through the clinical course of invasive breast cancer, it is relatively congruent at all metastatic sites, and it is not affected by tumor heterogeneity (28). This has potential clinical applications because it may allow development of therapies based on HER-2 targeting (28).

Previous studies have shown a direct relationship between HER-2 overexpression and sensitivity to CTL of ovarian tumors (18). Since overexpression of HER-2 may induce expression of other proteins that can provide peptides (18) with the same or cross-reactive sequences, gene and protein sequence databases were searched for homologous sequences. 100% matches for both C85 and E75 were not found. For E75 only EGF-R (HER-1), HER-3, and HER-4 gave matches for the main HLA-A2 anchors at P2, P6, and P9, but nonconservative changes (underlined) were found in positions 1, 3, 5, and 7 (EGF-R, residues 364-372:SISGDLHIL, HER-3, residues 356-364:KILGNLDFL, HER-4:KINGNLIFL). Central positions are expected to be contact points for TCR (15, 29). Matches for peptide C85 appear in EGF-R, ERB.B3, DNA-directed RNA polymerase (RPB-1), and in an unknown nuclear protein (UL2-1). Nonconservative changes in the se-

quence are dominant at positions expected to be TCR contacts such as P4 and P7 in RPB-1 and P5 in UL2-1. Based on recent crystallography data, the peptide termini are bound to HLA-A2 similarly but the central area of the peptide adopts different conformations that represent the epitopes recognized by TCR (29). Therefore, the nonconservative changes in the sequence of the homologous peptides from the other members of the HER family may affect epitope conformation and if these peptides are processed, presented, and recognized by TCR may constitute the equivalents of variants of peptides derived from the HER-2 protein.

As for the other tumor Ags (10, 25, 26), validation of HER-2 epitopes requires identification and quantitation of peptides bound to HLA class I on ovarian tumors. Since E75 lacks charged residues in the central area, it will be important to determine whether the same or conservatively substituted peptides from other proteins are naturally processed and presented to CTL. With the exception of E75 recognized by all four $CD8^+$ CTL, and in part of C85, which confirms our previous findings with unseparated CTL-TAL (20), three CTL recognized distinct HER-2 peptides at low level. These peptides were different in each system and their HLA-A2–stabilizing ability was variable over a wide range of concentrations. E89 binding affinity to HLA-A2 is at least three to four orders of magnitude lower than of naturally processed viral peptides (12), suggesting that the affinity of TCR for E89–HLA-A2 complexes may be high. This may also be true for C85. The affinity of a peptide for HLA-A2 is not the determining factor for the abundance of particular peptide presented by HLA-A2 (30). Other important factors are protein concentration and the processing efficiency of an antigenic peptide (30).

The mechanisms of HER-2 overexpression reflect gene amplification and upregulation of transcription (31). The involvement of translational, posttranslational mechanisms, or reduced rate of HER-2 turnover in HER-2 overexpression in cancer cells are still unclear (31). At this time, there is no simple explanation for the distinct pattern of peptide recognition between these lines. All tumors autologous with these CTL overexpressed HER-2 protein at similar levels that were consistent with HER-2 receptor overexpression when analyzed using monoclonal antibody *Ab2* that is specific for the extracellular domain of HER-2 (e.g., the levels of HER-2 expression were similar for OVA-1 and OVA-4 tumors [autologous with CTL-1 and CTL-4]: OVA-1, 79% $HER\text{-}2^+$ cells, MFC = 29, OVA-4, 100% $HER\text{-}2^+$ cells, MFC = 34, control OVA-17, 21% $HER\text{-}2^+$ cells, MFC = 4). However, CTL-4 associated with OVA-4 recognized in addition to E75 three other peptides. It is possible that these peptides if presented are processed by the tumor with different efficiencies (30). It is also possible that while for self-proteins the tolerance is not absolute, as shown for melanoma TIL specific for either MART-1 or gp100 (10, 25), elimination of high affinity T cells for a number of epitopes by tolerance may affect CTL with distinct specificities in each individual (10, 25).

Processing of overexpressed HER-2 in cancer cells may lead to peptides that differ in quantity from the HER-2 epitopes found on normal cells. Since HER-2 is present in normal epithelial tissues at lower levels and the protein concentration may be a limiting factor in epitope presentation, it will be important to determine how widely CTL-mediated HER-2 recognition is observed in ovarian and breast cancer patients, and whether such CTL can cause tumor rejection and show toxicity towards normal tissues.

This work was supported by National Institutes of Health (NIH) grant CA-57293. The peptide synthesis was supported in part by NIH core grant CA-16672.

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Received for publication 6 September 1994 and in revised form 13 February 1995.

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Molecular Immunology 35 (1998) 1121–1133

Molecular Immunology

Ovarian and breast cytotoxic T lymphocytes can recognize peptides from the amino enhancer of split protein of the *Notch* complex

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Received 27 April 1998; accepted 30 September 1998

Abstract

In this study we investigated recognition by ovarian tumor associated lymphocyte (OVTAL), and breast tumor associated lymphocytes (BRTAL), of peptides corresponding to the sequence 125–135 of the Aminoenhancer of split (AES) protein. Three of these peptides designated as G75:AES1/2 (128–135), G60: AES1/2 (127–137) and G61: AES1/2 (125–133) correspond to the wild-type AES sequence, while the fourth G76:GPLTPLPV, AES1/2 (128–135) corresponds to a variant sequence of the peptide G75 with the N-terminal Leu substituted to glycine. These sequences were chosen for study because mass-spectrometric analysis (MS) of a CTL active HPLC peptide fraction eluted from immunoaffinity precipitated HLA-A2 molecule, revealed: (a) the presence of an ion with a mass-to-charge ratio (m/z) of 793 which was more abundant than other ions of similar masses; (b) the tentatively reconstituted sequence of the ion 793 matched the sequence of peptide G76. We found that AES peptides G75 (128–135) and G76 (128–135) (L128G) reconstituted CTL recognition at concentrations ranging between 200–500 nM. These concentrations are lower than concentrations reported to activate effector function of CTL recognizing other epithelial tumor Ag. Furthermore, analysis with cloned $CD8^+$ T cells indicated that G75 and G76 were not cross-reactive specificities, suggesting a key role for the N-terminal residues of the variant peptide in dictating specificities. Since the AES proteins are part of a set of transcriptional repressors encoded by the Enhancer of split [E(spl)] genes, and since these repressors are activated to suppress cell differentiation in response to *Notch* receptors signalling, the AES peptides may represent a novel class of self-antigens that deserve further consideration as tumor Ag in epithelial cancers.

Keywords: Notch; AES; CTL; Epitopes; Breast; Ovary

1. Introduction

Advances in diagnostic and conventional therapies have led to earlier detection and improved quality of life for cancer patients. However, the establishment of drug resistance has raised the need for novel approaches to therapy of tumors. During recent years, studies on human cancer antigens (Ag) have identified peptides from self-proteins that are recognized by cytotoxic T lymphocytes (CTL). Most of these antigens have been discovered in the melanoma system (Houghton, 1994, Boon van der Bruggen, 1996). The expression of these CTL epitopes has been found to be dependent in some instances but not in others on the levels of MHC-class I expression (Rivoltini et al., 1995; Fisk et al., 1997). Regardless of the presence of CTL in tumor infiltrations, the disease progresses suggesting that this CTL response is too weak to mediate tumor regression. Furthermore, tumor progression may be dependent on an immunoselection process, characterized by the fact that tumor cells that lack expression of certain antigens may gain a proliferative advantage (Seung et al., 1995; Kono et al., 1997). Thus, the elimination of tumor cells expressing

Abbreviations: OVTAL, ovarian tumor associated lymphocytes; BRTAL, breast tumor associated lymphocytes; CID, collision-induced dissociation; AES, Amino enhancer of split; TLE, Transducin-like enhancer of split; PCR, Polymerase chain reaction; m/z, mass-to-charge ratio; U, mass unit; MS, mass spectrometry; w.t., wild-type; MCF, mean channel fluorescence.

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0161–5890/99/$ - see front matter
PII: S0161-5890(98)00100-X

defined epitopes should allow unaffected growth of other tumor cells that do not express these epitopes.

Ag specific cancer vaccines may provide a complementary approach to traditional therapies if efficient targeting of cytotoxic effectors can be accomplished. In contrast with melanoma (Kawakami et al., 1994a,b; Castelli et al., 1995; Cox et al., 1994), there is little information on the nature of tumor Ag present on epithelial tumors such as breast and ovary which affect a large segment of the population. To this moment, the spectrum of tumor Ag and corresponding CTL epitopes in breast and ovarian cancer is limited. It includes mainly the deglycosylated Muc-1 core peptide epitope (Jerome et al., 1993, Ioannides et al., 1993a), and HER-2 epitopes, the latter detectable, in general, in tumors with HER-2 overexpression (Ioannides et al., 1993b; Fisk et al., 1995; Peoples et al., 1995; Yoshino et al., 1995).

Characterization of additional tumor epitopes are needed since it may allow development of polyspecific cancer vaccines, which can target a larger population of antigenically distinct tumor cells. Identification of such epitopes on epithelial tumors appear to encounter difficulties. Muc-1 and HER-2 were initially targeted for study because of their different post-translational modification (Muc-1) or overexpression (HER-2) on tumor cells compared with normal tissues (Ioannides et al., 1995; Peoples et al., 1995). There is little information on other tumor genes and proteins expressed on cancer cells that can provide the focus of study of CTL recognition using synthetic peptide mapping.

An additional difficulty in characterization of novel cancer antigens rests in the limitations in the availability of primary tumor in the large amounts ($>10^{10}$ cells) needed for biochemical characterization of extracted peptides (Cox et al., 1994; Slingluff et al., 1993; den Haan et al., 1985; Udaka et al., 1992). This leaves, at this time, as the only feasible approach for novel tumor Ag identification, the use of tumor lines as primary source of tumor peptides. Mapping of active peptide fractions from acid treated tumors using breast and ovarian CTL isolated from tumor infiltrating lymphocytes (TIL) may allow focusing the search on candidate CTL epitopes. The fact that these CTL are not induced or expanded by stimulation with the tumor line should allow identification of pre-existing epitope specificities in the patient. These peptides can then be sequenced by mass-spectrometry (MS) and the candidate sequences derived from integration of resulting daughter ions tested as synthetic peptide equivalents to induce activation of CTL effector functions.

We have recently used this approach for characterization of the common peaks of naturally processed peptides shared between an ovarian tumor line (SKOV3.A2) and a freshly isolated ovarian tumor. We found in addition to a number of overlapping peaks of biological activity, several non-overlapping peaks of activity (Fisk et al., 1997a,b). The presence of the overlapping peaks is of interest because it suggests that such epitopes may have been presented on the original primary tumor and were stimulators for CTL.

MS analysis of the ions present in the peak *B*2 of overlapping activity corresponding to the HER-2 peptide E75 (Fisk et al., 1997a,b) revealed the presence of a number of ions (Fisk et al., 1997b). The signal intensity of several ions in a number of fractions matched the pattern of CTL activity of two ovarian tumor associated CTL-TAL lines (Fisk et al., 1997b). One of these ions of *m/z*: 792.9 (and further designated as ion 793) was selected for sequencing by MS because its signal intensity was significantly higher than that of the other ions of similar or higher masses suggesting an abundant peptide. Reconstitution of the 793 sequence suggested several possible peptides, of which, the best match 7/8, was found within the sequence responsive amino acids 128–135 of the amino enhancer of split protein (AES-1/2) (Miyasaka et al., 1993) of the *Notch* complex (Stifani et al., 1992) associated with cell differentiation (Artavanis-Tsakonas et al., 1995). Synthetic peptides of these sequences were found to reconstitute recognition of two ovarian and two breast TAL lines, isolated from ascites or pleural effusions respectively, suggesting that they may provide an additional target for tumor specific CTL.

2. Materials and methods

2.1. Cells and cell lines

The ovarian tumor line SKOV3.A2 has been previously described (Fisk et al., 1995). Other targets used in these studies consisted of freshly isolated breast and ovarian tumors from malignant effusions. BRTAL and OVTAL ascites or pleural effusions occurring in patients with advanced breast or ovarian carcinomas were isolated from ovarian ascites (OVA-TAL) or breast pleural effussions and ascites (BRTAL). Isolation of tumors, lymphocytes and lymphocyte culture was performed as previously described (Fisk et al., 1995). CTL assays to determine recognition of peptide pulsed T2 cells, tumor lysis and cold-target inhibition assays followed the previously reported procedures (Fisk et al., 1994, 1995). Tumor peptide extraction, HPLC fractionation using two acetonitrile gradients and CTL epitope reconstitution assays have been described (Fisk et al., 1997a,b). Effectors were generated by culture of OVTAL and BRTAL in RPMI media containing 10% FCS and 50 U/ml (Cetus) of IL-2 (complete RPMI medium).

For separation of $CD8^+$ cells, freshly isolated OVTAL and BRTAL were propagated in RPMI 1640 medium containing 10% FCS, antibiotics and 50 U/ml of IL-2 (Cetus) for one week. Afterwards the $CD8^+$ cells were isolated using magnetic beads (Dynabeads, Dynal, Oslo,

Norway) and cultured with the same conditions. For the purpose of limited cloning, the $CD8^+$ cells were plated in 96-well plates using binary dilutions ranging from 20–5000 cells/well in the presence of irradiated PBMC from HLA-$A2^+$ donors and alternatively stimulated with OKT3 mAb and PHA. None of these cultures was stimulated with peptides or tumor cells. Furthermore, tumor cells were not used as feeders. Two to three weeks later, the wells were scored for growth. Using this procedure, we found that in most instances proliferating cultures of $CD8^+$ cells resulted from wells initially seeded with 80–160 cells/well but not from wells where the $CD8^+$ cells were seeded at lower densities (Ioannides et al., 1991). Thus we assumed that from wells containing 80 cells or more, at least one $CD8^+$ cell was able to proliferate, while such a $CD8^+$ cell was absent from wells containing half the cell number (i.e. 40 cells) because proliferation was not observed from cultures started with 40 $CD8^+$ cells/well. Of the 'clonal' cultures by the above approach, the ones that maintained stable growth for at least one month from the initiation of the limited cloning procedure were tested for peptide recognition. Since these cultures were not recloned they are designated as T cell lines.

2.2. Mass-spectrometry

Five consecutive HPLC fractions (fractions 38–42) corresponding to the peak B2 of activity of peptides eluted from the immunoaffinity separated HLA-A2 molecules from SKOV3.A2 cells were analysed by MS for the presence of ions, whose relative abundance matched the CTL activity of two ovarian CTL-TAL lines (Fisk et al., 1997b). The single-charged ion of $m/z = 793$ was found in fractions 40 and 41, but not in the other fractions. Identification of the ion composition of the peak B2 fractions has been recently reported (Fisk et al., 1997b). Detailed methodological approaches to ion analysis and MS sequencing have been reported (Fisk et al., 1997b). Sequencing of the ion 793 was performed by the Analytical Biochemistry Center of the University of Texas Medical School in Houston, Texas. Collision-induced dissociation (CID) mass-spectra were obtained with a Finnigan MAT TSQ70-triple-quadrupole instrument upgraded with TSQ700 software and a 20 kV conversion dynode electron multiplier. For ion scans, the resolution of the first quadrupole (Q.1) was adjusted to allow transmission of +2 U from the center of the mass of interest. A peak width of 1 U was used for post-acquisition spectral averaging and quantitation by manual integration of selected chromatograms.

Sequence reconstitution was also performed using the computer program PEPSEQ version 1.2 (Sampson et al., 1995). Although this program identifies a large number of potential candidate sequences, it also focused the search for the candidate sequence by allowing increasing stringency. Peptide sequences were identified based on the concordance of determined and predicted mass-values for peaks of ions in a candidate sequence. The concordance was determined based on the lowest deviation between experimental and theoretical values for the respective ions and defined as the lowest score/peak ratio (Fisk et al., 1997b).

2.3. Synthetic peptides

Synthetic peptides of candidate ions prepared were: G76 (AES-1: 128–135,GPLTPLPV), G75 (AES-1: 128–135, LPLTPLPV). The peptide of the same sequence with G76 with the last two C-terminal residues inverted was designated G57:GPLTPLVP. To examine the possibility that the epitope formed is part of a longer peptide, the following peptides were prepared by extending the sequence AES-1,128–135, by two residues at C-terminus, i.e. G58:GPLTPLPVGL, G59:PLTPLVPGL, G77:GPLTPLPVGL and G78: PLTPLPVGL. To examine the possibility that the N-terminal extended sequence forms CTL epitopes two peptides were prepared G60: (AES-1, 127–135): ALPLTPLPV, and G61:(AES-1, 125–133): ALALPLTPL. The numbers assigned for the position of these peptides in the sequence follow the sequence of AES-1 protein. The sequence in the AES-2 protein is identical in this area, but the position of the sequence is N-terminally shifted by 11 residues in AES-2 compared with AES-1(21). AES-1 and AES-2 proteins, resulted likely from alternative splicing of the same precursor mRNA (Miyasaka et al., 1993; Mallo et al., 1995). For clarity, only the AES-1 sequence is referred to in this study. These peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by HPLC to >95% purity. The codes used to identify peptide in this study were assigned by the same laboratory. Peptides G76, G75, and G57 were sequenced by CID as described above, and their fragment ions spectra were compared with the ions resulted from the natural ion 793. To facilitate presentation, the amino acids substituted from the natural sequence or groups reverted from the natural sequence are underlined. All other peptides used in these studies have been previously described (Fisk et al., 1995).

2.4. Immunofluorescence

HLA-A2 stabilization assays were performed using the T2 line as indicator as we previously described (Fisk et al., 1996). Expression of HLA-A2 was determined using the HLA-A2 specific mAb, BB7.2 and MA2.1. Hybridomas secreting these mAb were obtained from ATCC. The stabilizing ability of AES peptides indicative of their HLA-A2 affinity was determined from their ability to enhance HLA-A2 expression after overnight culture with T2 cells as described (Fisk et al., 1996).

3. Results

3.1. Characterization of the sequence of the ion 793

The ion 793 was sequenced by mass-spectrometry using collision induced dissociation (CID). To obtain a candidate peptide sequence, the resulting daughter ions were first examined using the program PEPSEQ version 1.2. To focus the search for a sequence, the stringency was increased after each round of analysis. At a tolerance of 0.5 U (<0.1% deviation from the mass of ion 793) only six candidate sequences were selected by the PEPSEQ program. A search in nucleic acid and protein data bases using candidate peptides containing in the sequence either Leu (L) or Ile (I) or both revealed that of the six sequences only the sequenceGPLTPLPV gave the highest number of matches (7/8 matches) with a known sequence LPLTPLPV. This sequence corresponded to amino acids 128–135 of the amino enhancer of split (AES) protein. The gene for this protein is a member of the *TLE* (transducin-like enhancer of split) genes (TLE complex) associated with the *Notch* complex (Miyasaka et al., 1993). The human AES and TLE-1,-2, and -3 proteins show significant sequence homology in other areas but differ in this particular area (e.g. human TLE-1:139–150 sequence is GPPVPLPHPSGL (Stifani et al., 1992).

To characterize the correspondence between the experimentally determined and predicted sequence for ion 793, synthetic peptides G76:GPLTPLPV. G57:GPLTPLVP, and G75:LPLTPLPV were prepared. These peptides were then sequenced by CID. The predicted mass values for the *y* and *b* ions of peptide G76 are listed below (Table 1). These values were highly similar to the values predicted for peptide G57 ions, in which the last two C-terminal amino acids are reverted from the sequence of G76 (data not shown). Sequencing of peptide G76 showed a good correlation between the experimentally obtained and predicted mass values for 5/8 *b*, 4/8 *y*, 2/8 *bo*, 4/8 *yo*, and 2/8 *a*, G76 daughter ions (Fig. 1A). Because the amount of HPLC sample used for sequencing was limited, to integrate the sequence the resulting daughter ion masses of the peak 793 were compared with respect the position and signal intensity with the predicted masses of the G76 daughter ions *b* and *y* and the corresponding *a*, *bo*, and *yo* ions (Papayannopoulos, 1995). The experimentally obtained sequence data for peptide G76 and ion 793 are shown side by side in Fig. 1A. Although some major daughter ions species of peptide G76 were not detectable in the 793 spectrum, seven of eight *b* ions and four of eight *y* ions of 793 were present and found to match within 1 U, with the predicted values of the corresponding ions for peptide G76:GPLTPLPV. Furthermore, peaks corresponding to 4/8 *yo* ions, 3/8 *bo* ions and 2/8 *a* ions of the peptide G76 were also present in the spectrum of fragment ions of 793 (Fig. 1A).

To identify a candidate peptide sequence for the ion of *m/z* 793 we used in the interpretation of the data first the candidate *b* ions, then the candidate *y* ions. Expanded CID spectra for the ion of *m/z* 793 are shown in Fig. 2A and 2B. These spectra were obtained by normalizing the data to the next most abundant ions other than *m/z* 496 and 793 which were the most abundant (Fig. 1A). This allowed the presence and position of the smaller peaks to be determined more accurately.

From integration of the values for the candidate *b* ions *m/z* (58.2, 155.5, 369.3, 465.3, 577.0, 679.1 and 775.2) the candidate sequence appears as GPZZPLVP, (Z symbolizes an unknown residue). Then we examined the candidate *y* and *yo* ions. *b* and *bo*, *y* and *yo* ions differ in mass by a molecule of water (18 U). Thus from *y*/*yo* ions of *m/z* 793/775, –/717, 639/621, 526/508, 328/–, 216.9/200 (?) the first three N-terminal residues can read as GPL, while the last two C-terminal residues may read PV/VP (See also candidate ion $y2^{1+}$ *m/z* 216.9 and yo2 *m/z* 199–200. Based on the difference in mass between candidate *b*4 and *b*2 ions [369.3–155.5 = (213.8)] if Leu or Ile is present at P3, then the P4 residue may be Thr (Papayannopoulos, 1995). Thus integration of *y* ions led again to a candidate peptide of sequence either GPLTPLPV or GPLTPLVP (Papayannopoulos, 1995). Our data do not allow for unambigous assignment of the order of the last two amino acids. Therefore, the sequence of ion 793 deduced from *b* and *y* ions also shows a good match (7/8) with the sequence of AES-1/2 proteins in the area 128–135, initially tentatively identified using the PEPSEQ program. The signal for N-terminal Leu (mass 113) corresponding to the wild-type AES peptide was not detected in the spectrum of the ion 793 but was detectable in the spectrum of the synthetic peptide G75 suggesting that its

Table 1
Predicted masses of daughter ions of the peptideGPLTPLPV: AES-1 (128–135)*

No.	Seq.	*a*	*b*	*bo*	*d*	*y*	*yo*	No.
1	Gly	30.0	58.8	40.0	75.1	793.5	775.5	8
2	Pro	127.1	155.1	137.1	172.1	736.5	718.5	7
3	Leu	240.2	268.2	250.2	285.2	639.4	621.4	6
4	Thr	341.2	369.2	351.2	386.2	526.3	508.3	5
5	Pro	438.3	466.3	448.3	483.3	425.3	407.3	4
6	Leu	551.4	579.4	561.3	596.4	328.2	310.2	3
7	Pro	648.4	676.4	658.4	693.4	215.1	197.1	2
8	Val	747.5	775.5	757.5	792.5	118.1	100.1	1

*The values in the vertical columns indicate the residue masses of the peptides resulting from degradation of peptide G76, in the free amino and free acid form, starting from each terminus. The *bo*/*yo* ions differ from the corresponding *b* and *y* ions by the loss of one molecule of water (i.e. 58 – (16 + 2) = 40), while the *a* ions differ in mass from the *b* ions by the loss of a carbonyl group i.e. 58 – (16 + 12) = 30). The exact mass of peptide G76 (C38 H64 N8 O10) is 792.47. The exact mass of peptide G57 with the C terminal Pro-Val group reverted as compared to G76 is the same with G76.

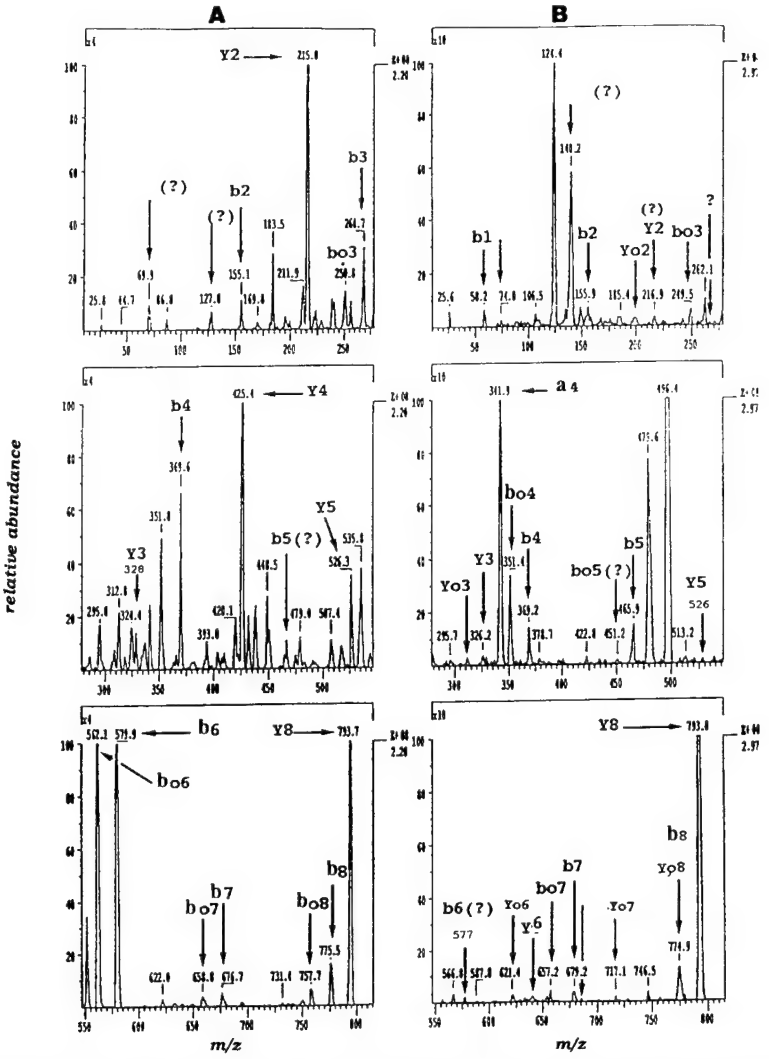

Fig. 1. CID spectra of the peptide G76 **(A)** and of the ion 793 **(B)**. The ions with similar positions are marked with arrows. The tentative assignment of the ions was made by comparing the experimental values with their predicted values listed in the Table 1. The ions which differ from the predicted values by more than 1.5–2 U are indicated with the mark(?). Note the absence of *b*1 and *y*1 from the spectrum of peptide G76 and the presence of the strong ionizing ion 496.4 in the spectrum of the ion 793. The experimental conditions were as described in the Materials and methods.

lack of detection does not represent a limitation of the sequencing method (data not shown).

The CID spectrum of the ion 793 also shows the presence of strong signals from ions *m/z* 124, 140, 496, as well as of signals from other ions at weaker intensity. The first three ions 124, 140, 496.4 may represent impurities (likely phospholipids, unpublished observations). This is because MS/MS spectra from other ions *m/z*: 453, 609, 638, 787, 1008 and 1017 detected in this peak (Fisk et al., 1997) showed a similar pattern of background interference. For example the ion 140 was found in the CID spectra of ions of *m/z* (453, 609, 638, 787, 1008, and 1017), the ion 124 was present in the CID spectra of ions *m/z* 609 and 638, while the ion 496 was present in the CID spectra of the ions of *m/z* 609, 638, and 786 (data not shown).

It should be noted that these peptides were isolated as mixtures from very complex biological matrices and were not expected to be pure. Ions 124 and 140 did not correspond to dipeptides. Ion 496 was the ion with the strongest intensity in the spectrum, stronger than that of any *b* and *y* ion and even stronger than that of ion 793. Some of the other ions present may indicate the presence of an additional peptidic component at lower density, e.g. ions 279/261, 479, 566/538, and 653. Thus, at this time we cannot rule out the possibility that the active fractions contain a mixture of active and inactive components of peptide nature not all of which correspond to AES sequences.

Clarification of the presence of the missing ions in 793, and certification of its peptidic nature would have required additional material which would in turn have meant growing more tumor cells on an even larger scale ($>40 \times 10^{10}$ cells). However, the important question was

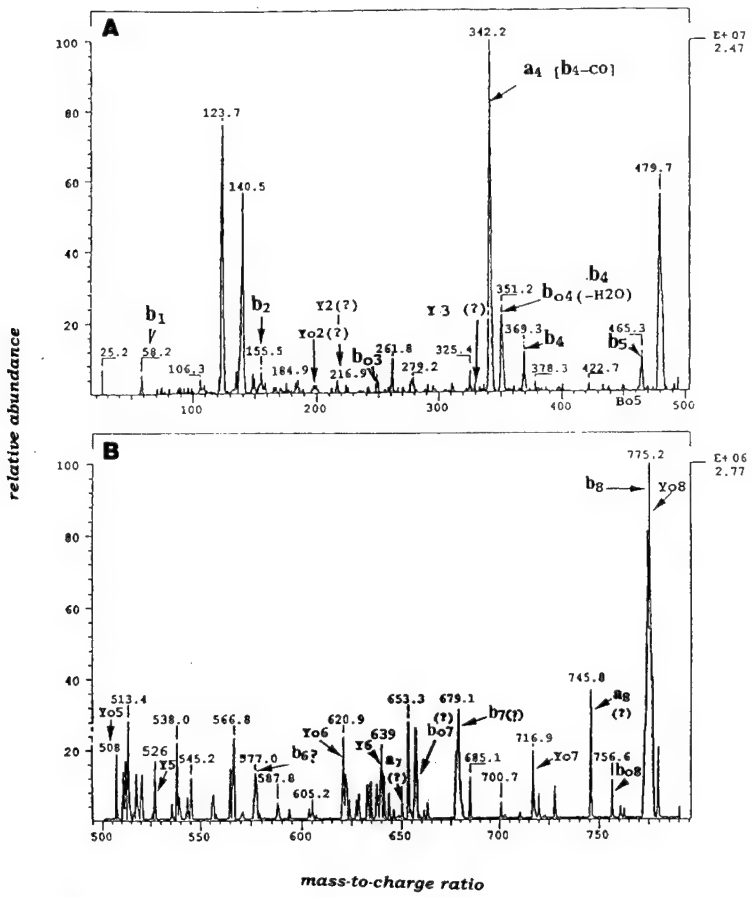

Fig. 2. Enlarged presentations of the CID spectra of the ion 793, after leaving out **(A)** the ions of m/z 496.4 and **(B)** the ion of m/z 793.0. Arrows indicate the positions and designation of the candidate Y and B ions by comparison with the values in Table 1. Note the low signal for the ions in the expected positions for $b1$, $b2$, $b3$, and the absence of $y1$, $y3$, $y4$ and $y7$.

whether a peptide with this sequence is functional, and the significance of its function. We hypothesized that if the proposed sequence for the ion 793 corresponds to a peptide, and this peptide is functional then it should be able to: (a) bind to HLA-A2 and (b) activate effector pathways by $CD8^+$ T cells. Therefore to establish the significance of the ion 793 and its related peptides, we decided to investigate recognition by breast and ovarian CTL of AES peptides encompassing the amino acids 125–135.

The area AES-1, 125–135 includes two overlapping sequences ALPLTPLPV (127–135) and ALALPLTPL (125–133) that contain HLA-A2 binding motifs and strong P1P2 anchors. Since the proposed sequence for the ion 793 corresponded to an N-terminally mutated AES peptide of the sequence 128–135, we prepared in addition to G76(GPLTPLPV) and G75(LPLTPLPV), the peptides G60(AES-1 (127–135 ALPLTPLPV) which differs from G75 by the presence of N-terminal Ala, and the peptide G61 (AES-1 (125–133) which overlaps in part (LPLTPL) with the other three peptides. It should be noted that the m/z of AES-1 peptides, 128–135 (designated as G75), 127–135 (G60), and 125–133 (G61) are different and higher than 793, e.g. m/z of G75 = 849.1. HPLC analysis indicated that they elute with distinct retention times (data not shown). Thus it is unlikely that they are present in the same HPLC peak.

3.2. Stabilization assays

To address the question of the ability of these AES peptides to bind HLA-A2 we performed T2 stabilization assays. The results in Table 2 show that, with respect reactivity with BB7.2 mAb, the epitopes formed by peptides G76 and G75 are likely to be conformationally different (Fisk et al., 1996). Staining with MA2.1 mAb revealed a higher stabilizing ability of HLA-A2 by G76 than did staining with BB7.2 mAb. In contrast, the levels of HLA-A2 stabilization detected by MA2.1 and BB7.2 mAb were similar for the peptide G75 corresponding to the wild-type AES-1 (128–135) sequence. The possibility that G75 and G76 are conformationally different epitopes was strengthened by the fact the peptides G77 and G78 (AES-1, 128–137) which differ from G76 and G75 by the presence of the C-terminal group Gly-Leu, induced a similar pattern of staining with G76 and G75 by MA2.1

Table 2[a]
Stabilization of HLA-A2 expression on T2 cells by AES peptides

Code	Sequence	MCF-R[a] MA2.1	BB7.2
G76:	G P L T P L P V	1.76	1.03
G75:	L P L T P L P V	1.53	1.52
G77:	G P L T P L P V G L	1.51	0.96
G78:	L P L T P L P V G L	1.44	1.2
G60:	A L P L T P L P V	4.66	N.D.
G61:	A L A L P L T P L	3.06	2.80
G57:	G P L T P L V P	1.29	N.D.
G58:	G P L T P L V P G L	1.07	N.D.
G59:	P L T P L V P G L	1.58	N.D.

[a] represents the mean channel fluorescence ratio (MCF-R), between the MCF corresponding to T2 cells incubated with any of the peptides and T2 (control) that have not been incubated with peptide (T2/NP). Each peptide was used at a concentration of 20 μg/ml.

and BB7.2 mAb. C-terminal extension of the epitope (as in peptides G77 and G78) did not increase the binding ability of the peptide to HLA-A2. The stabilizing ability of peptides G57 and G58 with the C-terminal group reverted to VP (P7–8) was lower than that of the corresponding w.t. peptides G76 and G77.

Peptides G60 (127–135) and G61 (125–133) showed significantly higher binding affinity for HLA-A2, than did all other peptides tested. The stabilizing ability of G60 was comparable with the stabilizing activity of the high-affinity HLA-A2- associated CTL epitope influenza-matrix (M:58-66) peptide. These results suggested that peptides containing the group GP at P1-2 can bind and stabilize HLA-A2 with low affinity even though they do not express the canonical HLA-A2 anchors. This also suggested that one candidate natural peptide(s) of the G76 sequence can be presented by HLA-A2.

3.3. Recognition of AES peptides by ovarian and breast tumor reactive CTL

AES sequence analysis indicate that in the same area (125–135), based on HLA-A2 binding motifs, several overlapping candidate CTL epitopes (G75, G60, G61) may be present. The L → G (128) change in the AES may reflect a variant member of the AES family as reported for other Ag (den Haan et al., 1995). This raised the question whether the w.t. peptide G75 and/or the variant peptide G76 are recognized by TAL. We decided to investigate in parallel the recognition of a w.t. candidate CTL epitope (represented by peptide G75), of the natural candidate CTL epitope (G76), and two overlapping nonamers containing canonical HLA-A2 anchors corresponding to the w.t. AES sequences 127–135 (G60) and 125–133 (G61).

Since ion 793 was found to be expressed by an ovarian tumor line, we first tested recognition of AES peptides by ovarian TAL. To determine whether any of these AES peptides are recognized by ovarian TAL (OVTAL) we first analysed the concentration-dependent recognition of G76 (w.t.), G76 (natural) and of the overlapping peptides G60 and G61. All these peptides shared the motif PLTPL. The effectors were generated by brief culture in medium containing IL-2 of OVTAL from HLA-A2$^+$ donors. These OVTAL were not restimulated with tumors during in vitro culture to avoid changes in Ag specificity. Both G75 and G76 were recognized at a concentration of 1 μg/ml by OVTAL-1 (Fig. 3A). Of interest, at 5 μg/ml G76 recognition indicated signs of saturation. Recognition of G60 and G61 was borderline and not significantly different from controls (data not shown). Since G75 and G76 differ only in the N-terminal residue we isolated CD8$^+$ lines from OVTAL-1, and tested recognition of G75, G76 as well as the lysability of SKOV3.A2 cells by these effectors. The results (Fig. 3B) show that the line OD8 recognized G75 but not G76. This line also recognized SKOV3.A2 cells suggesting that a similar epitope with the one formed by G75 may be present in the tumor cells. Another line, OF81, isolated from the same donor recognized G76 at concentration as low as 0.2 μg/ml but did not recognize G75 (Fig. 3C). The line OF81 also recognized SKOV3.A2 cells.

To evaluate whether OVTAL could recognize AES peptides G60 and G61, that bind HLA-A2 with high affinity, lysis of T2 cells preincubated with each of the low A2 affinity (G75, G76) and high affinity peptides (G60, G61) was tested in the same experiment over a range of concentrations by OVTAL from a second donor (OVTAL2). Both G76 and G75 were recognized by OVTAL-2 at lower peptide concentration (5 μg/ml) better than G60 and G61 (Fig. 4A–D). At 50 μg/ml recognition of G75 and G76 in fact decreased. G60 and G61 were recognized by OVTAL-2 although required significantly higher concentrations (25–50 μg/ml) than G75 and G76. Taking into consideration that G60 and G61 have significantly higher affinity for HLA-A2, than G75 and G76 this suggested that the affinity of TCR for the G76-HLA-A2 and G75-HLA-A2 epitope was higher than the affinity of TCR for the G60/G61- HLA-A2 epitopes. Since G75 and G60 differ only at their N-terminal Ala, this further indicates that the epitope(s) formed by G76/G75 were preferentially recognized over G60 and G61.

Similar results were observed when G75, G76, and G61 were tested for recognition by breast BR-TAL-1. G76 was better recognized than G75 and G61 (Fig. 5A–C). Therefore, the results, using two ovarian and one breast TAL lines, suggest that both octamers, the w.t. and the variant AES epitopes are recognized by TAL, with higher affinity than the overlapping nonamers G60 and G61. These results also suggest that epitope(s) recognized by the TCR of these TAL may be located closely to the N-

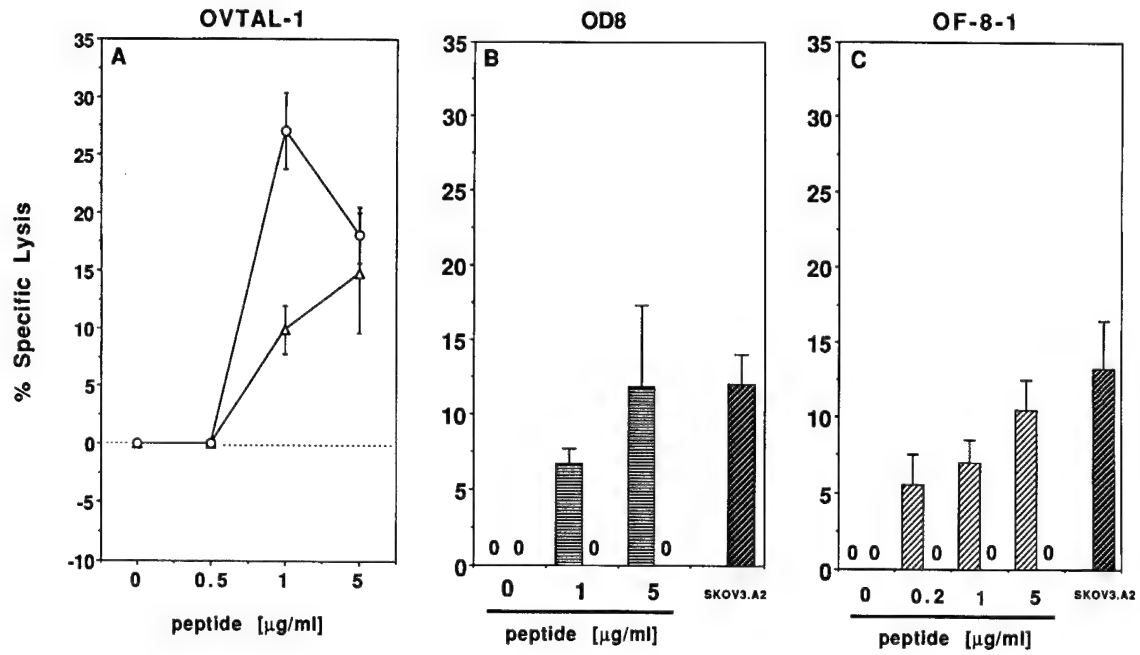

Fig. 3. **(A)** Concentration dependent recognition of AES-1 peptides G75 (0) and G76 (Δ) by OVTAL-1 at an E:T ratio of 10:1. Recognition of G75 (▤), G76 (▨) and of SKOV3.A2 (▨) by two $CD8^+$ lines, OD8 **(B)** and OF81 **(C)** derived from OVTAL-1. E:T was 8:1. Recognition by OVTAL-1, OD8 and OF81 was determined in separate experiments **(A)**. One of two experiments with similar results is shown. All experiments were performed in triplicate **(B)**, **(C)**. Recognition of G75 and G76 was significantly different from control, (T2/NP), no peptide pulsed T2 cells (O). Recognition of G76 by OD8 and of G76 by OF-8-1 was 0.0. ($P < 0.05$) by the unpaired Student *t*-test. OVTAL-1 recognition of peptides G60 and G61 was borderline and not significantly different from controls, and was not tested for lines OD8 and OF-8-1.

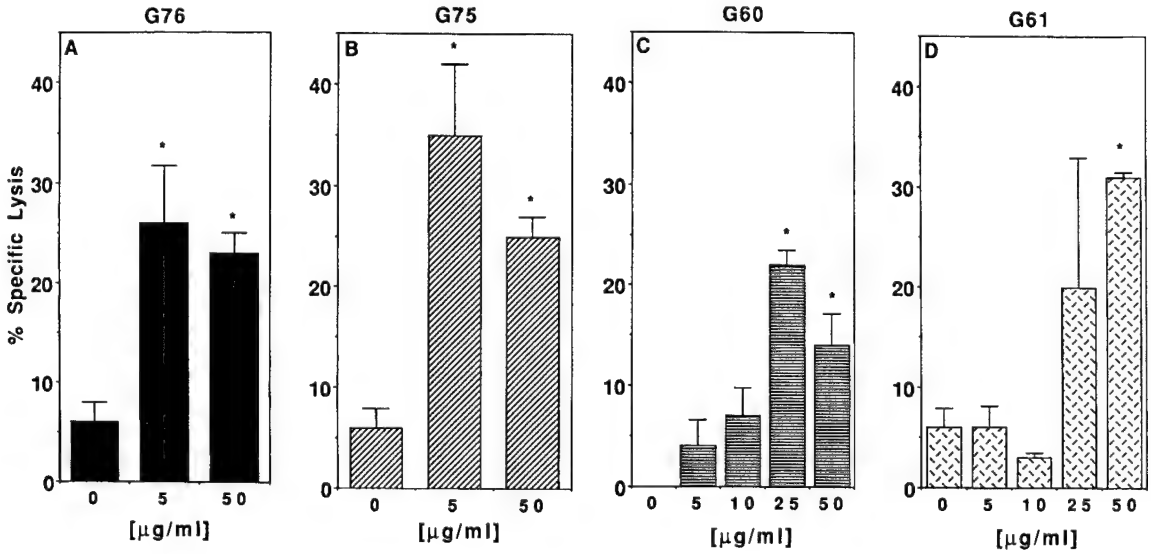

Fig. 4. Concentration dependent recognition of AES peptides G60 and G61 by OVTAL-2. T2 targets were pulsed with **(A)** G75, **(B)** G76, **(C)** G60, **(D)** G61. E:T ratio in the 5 h assay was 10:1 in all experiments. Recognition of G75, G76 and G61 was determined in the same experiment. 0, indicates T2/NP. Recognition of G60 was determined in a separate experiment performed three days later. Both G75 and G76 were recognized better than T2/NP ($P < 0.05$) at 5 μg/ml. One of two experiments performed in triplicate is shown. Results indicate mean ± SD. Recognition of G60 and G61 was significantly higher than of control (T2/NP) targets only at 25 and 50 μg/ml respectively. Experimental conditions were described in Materials and methods.

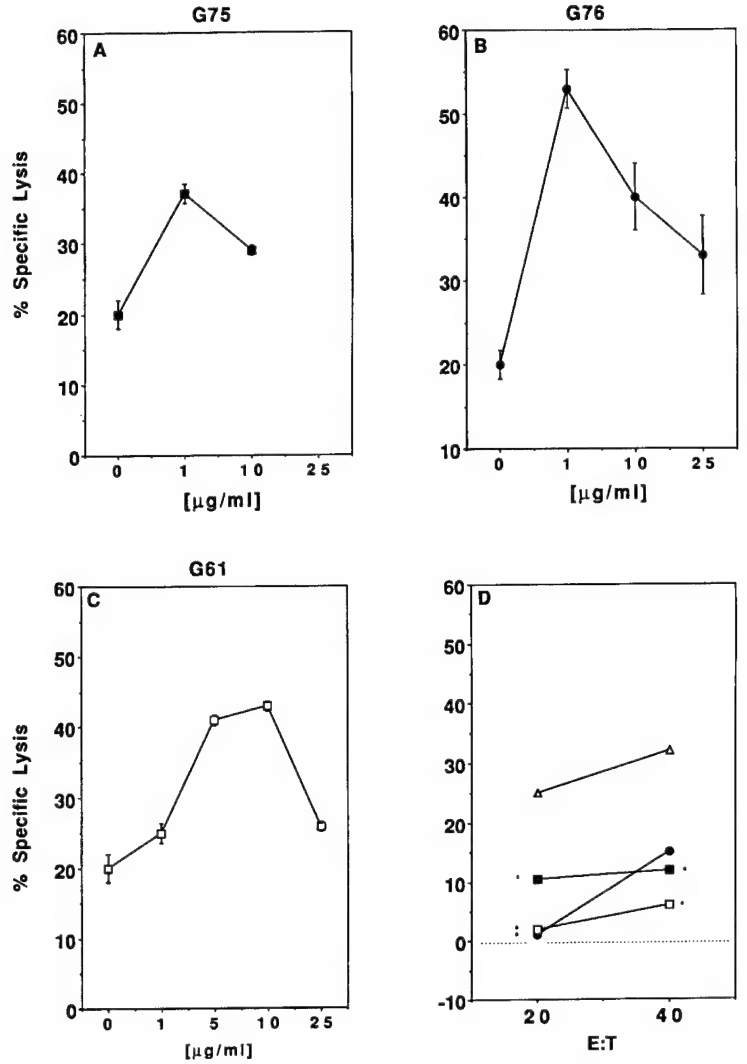

Fig. 5. **(A, B, C)** Concentration dependent recognition of AES peptides G75, G76 and G61 by BRTAL-1 in a 5 h assay. All determinations were performed in triplicate in the same experiment. Results respresent mean ± SD. E : T ratio was 20 : 1. Recognition of G75 and G76 was significantly higher than of control T2/NP (O) at 1 and 10 μg/ml. Recognition of G61 was not significantly different from control at 1 μg/ml. **(D)** Cold-target inhibition by AES peptides of the recognition of autologous tumor by BRTAL-1. All determinations were performed in the same experiment in triplicate. Results indicate mean ± SD. Because BRTAL-1 showed weak lysis of autologous tumor in 5 h assay (7.2%), the assay was continued up to 20 h to confirm the differences in inhibition of tumor lysis. T2 cells were pulsed with each peptide at 10 μg/ml. The cold : hot ratio was 10 : 1. The E : T ratio was 20 : 1 and 40 : 1. (Δ) NP, (●) G75, (■) G76, ( ) G61. (*) Significant inhibition of lysis was observed with G75, G76 and G61 at E : T ratio of 20 : 1 ($P < 0.05$) but only with G61 and G75 ($P < 0.05$), at E : T ratio of 40 : 1.

terminal area of these peptides, since all these peptides share the hexamer LPLTPL.

3.4. Cold-target inhibition experiments

To address whether AES epitopes are present on the autologous tumor with BRTAL-1, we performed cold-target inhibition experiments. All AES peptides tested inhibited recognition of autologous tumor by more than 50% compared with T2 cells pulsed with no peptide (T2/NP) at a cold : hot ratio of 10 : 1. The results in Fig. 5D also show that G76 was more effective than G75 in inhibiting recognition of the breast tumor by the autologous BR-TAL-1, suggesting that it is likely that an epitope similar to the one formed by G76 on T2 cells is expressed on autologous tumor. Autologous tumor lysis was inhibited even more efficiently than G75 and G76, by G61. It should be noted that BR-TAL-1 recognized more efficiently G75 and G76 than G61. The reasons for higher inhibition by G61 are not known, but G61 had significantly higher stabilizing ability for HLA-A2 than G75 and G76, thus it may be possible that a higher number of HLA-A2—G61 complexes are present on T2 cells over a longer period of time. These results also indicate that a peptide with similar or cross-reactive

sequence with G61 is presented by the freshly isolated metastatic breast tumor.

3.5. Both G75 and G76 specific CD8$^+$ cells are present in the freshly isolated breast TAL

The results presented above suggest that CTL specific for either G75 or G76 or both epitopes are present in the ovarian ascites and pleural effusions. To establish whether CTL specific for one of these peptides constitute a significant population, CD8$^+$ cells were isolated from a sample of breast pleural effusion (designated BRTAL-2) one week after culture in IL-2 and were further cultured using different starting numbers. When the cells in each culture were present in sufficient numbers to allow CTL assays they were tested for recognition of peptides G75 and G76. Of the 37 cultures tested, we found two cultures (15B and 27E) for which the levels of recognition of G76 were at least two-fold higher than of G75, and one culture (27F) for which the levels of recognition of G75, were at least two-fold higher than of G76 (Fig. 6A and 6B). Since these CTL were isolated from cultures initially seeded between 160 cells/well (27F, 27E) and 640 cells/well (15B), this suggests that the clonal size of G75-specific and G76-specific CTL should be significant.

To determine whether in these populations G76 specific clones are present, one of the lines, designated as 27E was recloned, and retested after expansion. The results in Fig. 6C show that the line B27E recognized G76 at both 0.1 and 1.0 μg/ml, at an E:T ratio as low as 2:1 but did not recognize G75. This confirmed the results obtained with the two ovarian TAL lines, suggesting that CD8$^+$ CTL of similar affinity and specificity for G75 and G76 are present in both epithelial tumor systems.

4. Discussion

In this study we have identified a novel candidate tumor Ag recognized by CTL present in the TAL from ovarian and breast tumors. This candidate tumor Ag consists of AES protein, which is part of the Notch complex involved in signalling for determination of cell fate during development and differentiation. The AES protein is proposed as a candidate tumor Ag based on the ability of several peptides of AES sequence to activate and inhibit the effector function of OVTAL and BRTAL.

These peptides present certain characteristics which have not been previously observed in other human peptides forming CTL epitopes:

(1) they derive from proline rich areas and contain in the sequence at least three Pro and three Leu residues;

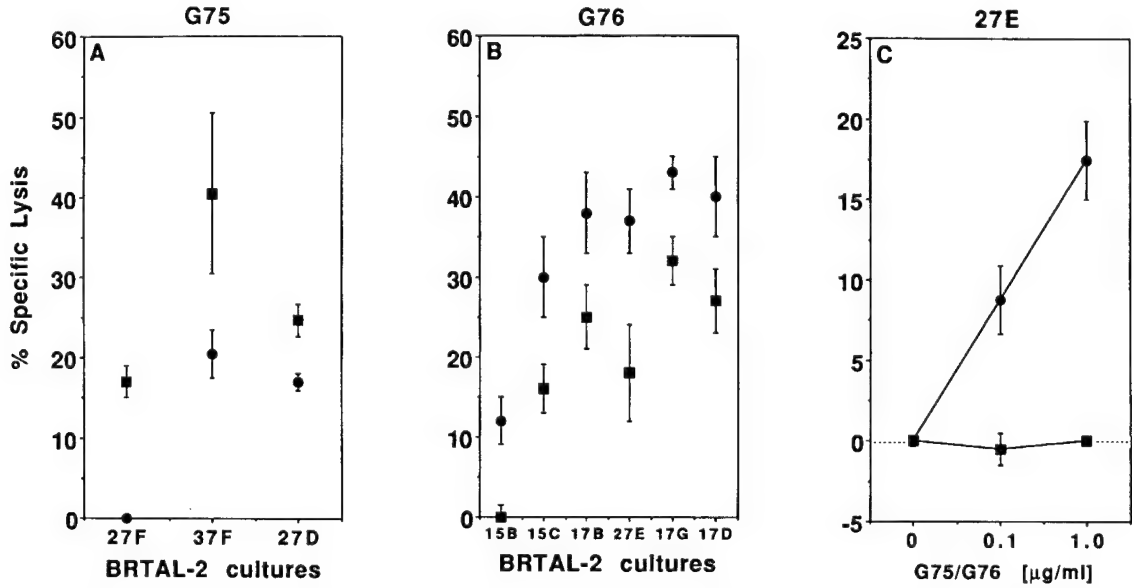

Fig. 6. **(A, B)** Patterns of G76 and G75 recognition by CD8$^+$ CTL isolated by limited cloning from BRTAL-2. **(A)** CD8$^+$ CTL cultures preferentially recognizing peptide G75. **(B)** CD8$^+$ CTL cultures preferentially recognizing G76. Target T2 cells were pulsed with 1 μg/ml of G75 and G76 respectively. Because of the large number of cultures to be tested for each culture, recognition of G75 (■) or G76 (●) was determined by an initial screening in the same experiment in duplicate. All resulting cultures were tested twice for recognition of G75 and G76. Only cultures which were confirmed in both experiments to preferentially recognize G75 or G76 were considered positive. The E:T ratio was 3:1. CD8$^+$ cells in the wells indicated as 5, 6, 7, 8 were initially seeded at 640, 320, 160 and 80 cells/well, respectively. Designations e.g. 27F indicate plate, column, row **(C)** Concentration- dependent recognition of G76 by line B27E isolated from a CD8$^+$ culture previously found in a separate experiment **(B)** to preferentially recognize G76 over G75. The E:T ratio was 2:1. (■) G75, (●) G76; **(C)** Recognition of G76 was significant compared with that of G75 at both 0.1 and 1.0 μg/ml ($P < 0.05$). The results show the means and standard deviations of one CTL assay performed in triplicate.

(2) two of these active peptides contain Pro at position 2 (P2). P2 corresponds to the main anchor position for peptide binding to HLA-A2. It is generally occupied by Leu/Ile and less frequently by Met, Ala, and Thr (Hunt et al., 1992);
(3) the most active peptides containing Pro at P2 were generally recognized with higher affinity, at 10-fold lower concentrations than the corresponding overlapping nonamers (AL)PLTPLPV containing canonical Leu (P2) and binding HLA-A2 with high affinity.
(4) One of the active peptides recognized with high affinity by ovarian and breast CTL, G76 corresponds to a fragment of a likely variant AES protein. This epitope is characterized by a Leu- > Gly change in the N-terminal residue, a change unlikely to be the result of a point mutation in the Leu codon, since no point mutations in the Leu codon can lead to the Gly codon (Stifani, S., pers. comm.).

In determining the recognition of the candidate natural epitope G76, we noted that its recognition peaked at 1–5 μg/ml and was inhibited at higher concentrations (25–50 μg/ml). This may reflect sometimes the presence of impurities in the HPLC purified peptides. For Pro-rich proteins, this may also reflect Ag aggregation/dimerization; This inhibitory activity was observed, at the same concentrations with peptide G75 which differs from G76 only at the N-terminal Leu. Although these results are preliminary since they were obtained with a small panel of effectors, they indicate that existing CTL recognize G75 and mainly G76 when pulsed on T2 targets at concentrations as low as 0.2–0.5 μg/ml (range 250–600 nM). These concentrations are significantly lower than the concentrations required to sensitize targets by most other peptides recognized by CTL in epithelial cancers (Fisk et al., 1995) and the majority of the melanoma tumor Ag (Houghton, 1994). Therefore, the possibility that G76 specific CTL are high affinity clones which are inhibited by high Ag concentrations, in a similar way with CTL recognizing classic Ag (Alexander-Miller et al., 1996) may deserve further investigation since it may provide a mechanism for tumor escape.

Based on cold-target inhibition studies, we surmise, that complexes structurally similar to the one formed by the AES peptides are present on the surface of tumor cells. Ongoing studies are attempting to clarify whether all three AES peptides are simultaneously presented by the tumor and the implications of Ag presentation from peptides containing Pro at P2.

AES peptide G76:GPLTPLPV was identified after MS sequencing of an ion with m/z 793 present in a HPLC fraction of peptides acid-eluted from immunoaffinity separated HLA-A2 molecules from a tumor line. This fraction reconstituted the CTL effector function of two OVTAL lines (Fisk et al., 1997a,b). The signal intensity of the ion 793 in two consecutive HPLC fractions matched the CTL activity induced by these fractions (Fisk et al., 1997b), thus it was considered a primary candidate for sequencing. Although our results neither provide certitude that ion 793 is a peptide, nor proof that it is the only active component of the peak 793, the approach we used may be useful for other Ag characterization studies. The extensive handling of samples used for Ag identification is accompanied by substantial losses in material (sometimes up to 90%) (Udaka et al., 1982), which need to be compensated by growing higher numbers of cells. The availability of small amounts of samples for peptide sequencing, does not allow a direct answer to the questions as to whether a particular ion present in an active peak has attached other groups (i.e. phospholipids, sugars) or even shorter peptides that may be active by themselves. In contrast, the focus on a candidate peptide and analysis of recognition of overlapping synthetic peptides may provide a rapid answer to the question whether a candidate tumor Ag, is recognized i.e. is/was immunogenic in vivo.

The critical tests of tumor Ag identification strategy are whether: (a) the candidate peptide is active in inducing effector function by T cells from the tumor environment and (b) the gene and its corresponding source protein are expressed by the tumor, i.e. CTL do not recognize cross-reactive species on other targets. Our results show that the AES peptides meet the criterion of activating of effector function. Ongoing studies in our laboratory have also found that peptides G75, and G76 but not G60 can induce IL-2 secretion by OVTAL, within 24h, suggesting that they can activate additional signal transduction pathways in effectors (Babcock et al., Manuscript in preparation). Furthermore, preliminary studies in our laboratory could not unambigously establish that (a) peptides with the group VP instead of PV: G59, G58, G59 and (b) peptides with extended C-terminal: G77, G78, G58, G59 are recognized by BRTAL and OVTAL. Thus, additional studies are needed to clarify these points. In the absence, at this time, of antibodies that unequivocally recognize human AES-1/2 in tumors and healthy tissues, we cannot quantitate the AES levels. Preliminary results in our laboratory using PCR and primers and probes specific for AES-1,2 indicate that AES transcripts are present in both the ovarian SKOV3.A2 and breast SKBR3.A2 lines, and they are distinct from the transcripts of the TLE-1-4 proteins. However, since the presence of a transcript does not always indicate the abundance of a protein, additional studies are needed to address this point.

The AES genes (also designated Grg) mapped to human chromosome 19 (Mallo et al., 1995), are part of the Enhancer of split [E(spl)] complex of genes, that also includes the similar TLE genes (Stifani et al., 1992). The E(spl) genes form a neurogenic locus in *Drosophila* (Artavanis-Tsakonas et al., 1995). The exact function of E(spl) proteins is not known, but it is possible that they function

as transcriptional repressors of effector genes for cell differentiation (Jarriault et al., 1995). These repressor pathways are induced by the activation of the membrane-bound Notch receptor and signal the suppression of differentiation. It was recently reported that TLE family members are overexpressed in combinatorial ways during differentiation of mouse embryonic carcinoma cells in vitro (Yao et al., 1998). The role of AES and its involvement in carcinogenesis or maintenance of undifferentiated state is unknown but the mouse analog Grg was implicated in inhibition of gene transcription (Mallo et al., 1995b). The AES proteins may be widely distributed in adult mice tissues (Mallo et al., 1995a) and possibly in human tissues (Miyasaka et al., 1993).

Four mammalian *Notch* genes (1, 2, 3, 4) have been identified (Shirayoshi et al., 1997). They are highly conserved relative to the Drosophila *Notch* gene and appear to be important for cell differentiation and neoplasia associated translocation (Larsson et al., 1994). Expression of *Notch*-1/*Notch*-2 proteins have been reported in adult tissues of the mouse including among others brain, thymus, spleen and lung (Ellisen et al., 1991), as well as human bone marrow $CD34^+$ stem cells (Varnum-Finney et al., 1998). Transcripts of human *Notch*-1 are abundant in human fetal tissues while overexpression or truncation of Notch-(Tan 1) are important determinants of oncotropic activity (Ellisen et al., 1991; Pear et al., 1996).

It may be tempting to propose that: (a) the synthesis of AES proteins also increase in cancer cells, to maintain the undifferentiated state as reported for TLE genes (Liu et al., 1995); (b) AES may be subjected to a more rapid turnover and/or interacts with another protein as recently reported for TLE proteins (Palaparti et al., 1997) and/or is misprocessed. (c) Overexpression of Notch-1 and Notch-2, as well as of the proteins of the TLE complex may result in overexpression and misprocessing of AES proteins. All these possibilities have been reported to lead to CTL epitope formation (Yewdell et al., 1996; Michalek et al., 1993). It remains to be seen whether AES is expressed in normal tissues, and whether the CTL recognizing these peptides also lyse healthy tissues. If the origin of ion 793 as well as the wild-type AES CTL epitopes will be confirmed, this may provide a novel Ag to target in cancer vaccination studies.

Acknowledgments

We thank Drs Victor Engelhard (University of Virginia), John D. Lambris (University of Pennsylvania) and Artavanis-Tsakonas (Yale University), for fruitful discussions and encouragement, and Mr Bruce J. Swearingen for preparation of figures. We also thank Ms. Susan Mondragon and Ms Yolonda D. Harvey for outstanding editorial assistance. This work has been supported in part by Grant DAMD 17-94-J-4313. Peptide synthesis was supported in part by the core grant CA 16672.

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ANTICANCER RESEARCH *19:* 2907-2916 (1999)

Folate Binding Protein Peptide 191-199 Presented on Dendritic Cells Can Stimulate CTL from Ovarian and Breast Cancer Patients*

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Abstract. *Tumor associated lymphocytes (TAL) isolated from malignant ascites cultured in media containing interleukin-2 show antitumor responses. These antitumor responses are mediated by cytotoxic T lymphocytes (CTL) which recognize antigen in the context of MHC molecules using T cell receptors. CD8+ CTL recognize peptide epitopes processed from cellular proteins in the context of MHC class I molecules. These peptides have a restricted length of 8-11 amino acids. The folate binding protein (FBP) is overexpressed in over 90% of ovarian and 20-50% of breast cancers. We recently found that FBP is the source of antigenic peptides recognized by a number of these CTL-TAL. This indicated that FBP peptides are antigenic in vivo for ovarian and breast CTL-TAL. To define FBP immunogenicity, a peptide defining the epitope E39 (FBP, 191-199) was presented by PMBC derived dendritic cells (DC) from healthy donors isolated by the CD14 method to ovarian and breast CTL-TAL. Stimulation of ovarian and breast CTL-TAL by E39 pulsed DC (DC-E39), in the presence of IL-2, rapidly enhanced or induced E39 specific CTL activity. This E39-responder population consisted of cells expressing TCR Vβ9, Vβ13, and Vβ17 families, based on the increase in the percentages of these families in DC-E39 versus DC-NP stimulated TAL. Characterization of immunogenic tumor antigens and of cytokine requirements for induction of functional antitumor effectors may be important for future cancer vaccine developments.*

*This work was supported in part by Grant DAMD17-94-3413 and the Women's Fund for HER.

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Key Words: Folate binding protein, FBP, T-cell receptor, TCR, cytotoxic T-lymphocytes, CTL, tumor associated lymphocytes, TAL, ovarian cancer, breast cancer.

The identification of tumor antigens (Ag) recognized by CTL in melanoma as well as in other cancers such as ovarian cancer has raised interest in developing novel molecular therapies for cancer based on tumor Ag stimulation of CTL [1,2]. Since the tumor Ag recognized by CTL consist of short amino acid sequences (8-11 residue long), which define epitopes presented by MHC-I molecules, the central hypothesis of all these studies is that these specific sequences can induce anti-tumor CTL immunity. Definition of the immunogenicity of these epitopes is based on their ability to stimulate CTL in vitro and in vivo to expand and express specific CTL function [3]. Although T cell stimulation and vaccination with short defined sequences is expected to overcome concerns of specificity of recognition and to focus the responses to well defined epitopes, tumor specific CTL stimulation/induction by short peptides has encountered difficulties [4-7]. This was expected given the reported complexities in inducing CTL capable of recognizing endogenously presented Ag, at stimulation with exogenously added nonamer peptides [8,9]. In general, exogenous peptides pulsed on various APC poorly stimulated CD8+ cells from PBMC, and lead to CTL that recognized at higher extent the exogenous but not the endogenous presented Ag. This phenomenon was more frequently described in extensive studies in the melanoma system, where patients vaccinated with wild type melanoma gp100 peptide 209-217, or with a mutated analog g9-209(2M) induced high numbers of peptide 209-217 specific CTL but weak clinical responses [10,11]. Further analysis demonstrated that such CTL recognized the wild type or the mutated analog, but poorly the gp100 expressing melanoma cells.

Similar results were obtained from *in vitro* and *in vivo* studies in other systems, such as ovarian carcinoma using

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HER-2 peptide E75 immunogen and incomplete Freund's adjuvant [12]. This was not unexpected since it has been previously shown in studies with tumor Ag that numerous repeated peptide stimulations are necessary for expanding Ag or tumor specific CTL from PBMC [13-14]. Furthermore, DC peptide stimulation can more easily expand activated [15] than primary effectors [16].

Ongoing studies are focussed on approaches to overcome the poor immunogenicity of the tumor Ag when delivered in peptide form. One of these approaches uses DC as APC. This aims to enhance the peptide immunogenicity by increasing both the Ag levels and the levels of co-stimulatory molecules. DC have the capacity to uptake higher amounts of peptides than other APC. While the DC approach appears to require less cycles of stimulation for CTL induction than the PBMC as APC, its use for therapeutic purpose depends on the availability of DC precursors. This is an important issue for cancer patients particularly for the ones with advanced disease with low blood counts and functionally impaired DC [17,18].

The fact that PBMC derived DC cultured in GM-CSF plus IL-4 show poor proliferation and limited life span [19], raises the possibility of using as APC, DC from healthy donors for stimulation of CTL. Tumor infiltrating lymphocytes (TIL) and/or TAL show a higher frequency of Ag-specific CTL than PBMC and consist of activated memory effectors [20]. This raises the possibility of stimulating TIL/TAL with peptide pulsed DC to expand Ag-specific clonal populations, on the rationale that lower Ag concentrations and less co-stimulatory interactions are needed for activation of memory than of naive T cells.

To investigate this possibility, we stimulated ovarian and breast TAL from six distinct patients with peptide pulsed allogeneic DC. The peptide used for stimulation correponds to a immunodominant CTL epitope mapping the amino acids, 191-199 of the FBP, a newly identified tumor Ag [21]. FBP is overexpressed in the majority of ovarian (over 90%) and 50% of breast cancers [22-25]. The DC were generated from healthy donors that were HLA.A2 matched with the patients. The results showed that E39 specific CTL could be easily generated from TAL from patients with advanced disease. These peptide E39 stimulated TAL recognized autologous tumors. DC-E39 stumulation lead to expansion of certain clones from the TAL population as illustrated by the preferential increase in population of CD8+ cells expressing certain TCR Vß families.

This increase was not due to allo-stimulation since TAL from the same patient stimulated with the same DC in the same experiment but in the absence of E39 failed to induce E39-specific CTL and specific increase of the same TCR Vß familes. These findings may be significant for therpeutic approaches for ovarian and breast cancer patients with advanced disease. This may be useful for *in vitro* immunization and expansion of CTL of desired specificity followed by adoptive immunotherapy.

Materials and Methods

Cytokines. The following cytokines were used in this study: GM-CSF (Immunex Corp., Seattle), specific activity 12.5 x 10^7 CFU/250mg, IL-4 (Biosource International), specific activity 2 x 10^6 U/mg, IL-2 (Cetus, Emeryville, CA), specific activity 4 x 106 BRMP U/mg, IL-15 (Genzyme, Cambridge, MA), specific activity 2 x 10^6 U/mg.

Synthetic peptides. Peptides were synthesized in the Synthetic Antigen Laboratory of U.T. M. D. Anderson Cancer Center using solid phase techniques on an Applied Biosystems 430 peptide synthesizer (Applied Biosystem, Foster City, CA). Identity and purity of final material were established by amino acid analysis and analytical reverse phase HPLC (Rainin). All peptides utilized in this study were between 92-95% pure. Two FBP peptides were selected for synthesis based on the presence of leucine, isoleucine or valine in the dominant anchors position. As their previously reported recognition by TAL the peptides position and sequence are as follows: E39 (FBP, 191-199) EIWTHSYKV; E41 (FBP, 245-253) LLSLALMLL. Both peptides are low to moderate binders to HLA-A2 [21].

Cells. For induction of dendritic cells in the presence of cytokines GM-CSF and IL-4, HLA.2+ PBMC were obtained from healthy donors from the Blood Bank of M.D. Anderson Cancer Center. For generation of DC by the CD14 method, PBMC were distributed in 24 well plates at 4 x 10^6 cells/well in RPMI 1640 medium. After 2 h of incubation, the non-adherent cells were removed. Complete RPMI medium containing 1000 U/mL GM-CSF and 500 IU/mL IL-4 was added to each well and the adherent cells were cultured for 5-7 days, while they developed the DC characteristic morphology.

Tumor Associated Lymphocyte Cultures. TAL were isolated from fresh collections of malignant ascites and pleural effusions from 4 ovarian and 2 breast cancer patients from the departments of Gynecologic Oncology and Breast Medical Oncology at U. T. M. D. Anderson Cancer Center under the approval of the Institutional Review Board. Specimens were processed as we described [26]. The suspensions of the lymphocytes and tumor cells were separated by centrifugation over discontinuous 75% and 100% Ficoll-Histopaque (Sigma, St. Louis, MO) gradients. Freshly isolated TAL were cultured in RPMI 1640 containing 100 (g/ml L-glutamine (Gibco, Grand Island, NY) supplemented with 10% FCS (Sigma), 40 (g/mL gentamicin (complete RPMI medium), and 50 to 100 IU/mL IL-2 (Cetus, Emeryville, CA). TAL were cultured at 0.5 to 1.0 x 10^6 cells/mL, placed in a humidified incubator at 37°C in 5% CO_2 and maintained at this concentration with the addition of media and IL-2 every 2 to 3 days, depending on the growth kinetics.

T cell stimulation by peptide pulsed DC. DC were washed three times with serum free medium, plated at 1.2 x 10^5 cell/well in 24-well culture plates and pulsed with FBP peptide, E39, at 100 µg /ml in serum free medium for 4 hours before addition of responders as described [27]. These DC were designated as DC-E39. Paralled control DC cultures were established and maintained in the exact same manner except for the omission of FBP peptide (designated DC-NP). The responder TAL in complete RPMI medium were added to DC at 3 x 10^6 cells/well (stimulator : responder ratio of 1:25). 16 hours later IL-2 was added to each well at a final concentration of 30IU/ml and the cultures were left undisturbed for the following 5 days when CTL activity was determined.

Tumor targets. The FBP+ ovarian SKOV3 line was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of

HLA-A2 expression (SKOV3. A2) as previously described [26] and maintained in complete RPMI medium and 250 μg/mL G418 (Sigma). Fresh tumors were collected from the malignant ascites after Ficoll separation and frozen in aliquots in liquid nitrogen until used.

Phenotype Analysis. The HLA-A2 status of the TAL lines and tumor cell lines was determined by indirect staining with anti HLA-A2 mAb BB7.2 (ATCC) followed by incubation with goat anti-mouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA) and analyzed on a Coulter Epics C Cytometer (Coulter Electronic, Hileah, FL). FBP expression was analyzed using the Mov18 mAb generously donated by Centocor (Malvern, PA).

Flow cytometry for TCR Vβ expression. TAL were stained with fluorescein and phycoerythrin-conjugated mAb specific for the TCR Vß families. The following mAbs were purchased from Pharmingen (San Diego, CA) and Endogen (Wolburn, MA). Vß3.1, Vß5a, Vß6.7, Vß8a, Vß9, Vß12, Vß13, Vß17, Vß23. The normal mouse IgG1 and IgG2a of Ig isotype were used as isotype controls. Two-color flow cytometry CD8:TCR Vß was performed using a FACScan (Becton-Dickinson) as described [28]. Since there are more than 20 Vß families, the average percent expression of each TCR Vß family should be in the range of 4-5%. We considered a significant increase in the percent Vß for each family when the difference between percent Vß of DC-E39 stimulated and DC-NP stimulated was higher than 5-10%.

Cytotoxicity assays. Recognition of peptides used as immunogens was performed by standard chromium release CTL assay as described [26,29]. T2 or tumor targets were labeled with 200 μCi of sodium chromate (Amersham, Arlington Heights, IL) for 1.5 hrs at 37°C, washed twice and plated at 3000 cells/well in 100 μl in 96 well V-bottom plates (Costar, Cambridge, MA). Effectors were added at designated effector : target (E:T) ratios in 100 μl/well. After 5 h of incubation, 100 μl of culture supernatant was collected, and ^{51}Cr release was measured on a gamma counter (Gamma 5500B, Beckman, Fullerton, CA). All determinations were done in quadruplicate. The results are expressed as percent specific lysis as determined by the equation : (experimental mean cpm - spontaneous mean cpm) / (total mean cpm - spontaneous mean cpm) x 100. For peptide-pulsed cytotoxicity assays, the T2 cells were labeled as above, washed, and then incubated either with PBS (T2-NP) or with peptides (DC-E39) for 1.5 hr at 37°C before standard CTL assays were performed.

Monoclonal antibody blocking assay. Effectors were incubated with anti-V beta mAbs Vß3.1, Vß5a, Vß6.7, Vß8a, Vß9, Vß12, Vß13, Vß17, Vß23. (50 μL of 1:50 dilution of culture supernatant/well) for 30 minutes at 37°C before being added to the standard CTL assays then the assays were performed as described above.

Cold target inhibition assays. Unlabeled T2 were incubated with E39 for 1.5 hr, then added to standard CTL assays with chromium-labeled tumor targets and effectors. The cold : hot target ratios were 10:1 and 20:1. The T2-NP were used as a control.

Results

Patients characteristics. Four ovarian and two breast cancer patients were selected for this study. The ovarian TAL (OTAL) and breast TAL (BTAL) were isolated from malignant ascites and pleural effusion specimens. They were all found to be HLA.A2+ and the CD8+cells in these ascites ranged between 20-40%. The patients ages ranged between 45 to 63 years, and the stages of diseases were all far advanced. According to cell types, all six patients had highly differentiated cell grade, and for the histology, two ovarian cancer were adenocarconoma, two were papillary serous cystadenocarcinomas and two breast cancers were infiltrating ductal carcinomas. They all received primary cytoreductive surgery follwed by adjuvant chemotherapies. The survival period for the study subjects ranged from 22-69 months for the ovarian cancer and 12-38 months for the breast cancers from the diagnosis of disease to study initiation. A brief description of patients chracteristics is summarized in Table I.

Table I. *The clinical characteristics of the patients.*

	Ovarian cancer			Breast cancer		
	Pt.1	Pt.2	Pt.3	Pt.4	Pt.1	Pt.2
Stage :	IIIc	IIIc	IIIc	IIIc	IIIb	IIIa
Age :	52	50	45	47	63	56
Histology :	AC	PSA	AC	PSA	IDC	IDC
Grade :	III	III	III	III	III	III
1st Tx :	TRS	TRS	TRS+BSO	TAH+BSO	MRM	RM
2ndTx :	PC	PC	PC	PAC	Taxol	CMF+CAP
Other Fx :		ER+,PR+	BrestCa		ER+,PR+	
Prognosis :	Poor	Poor	Fair	Poor	Poor	Poor

AC, adenocarcinoma; PSA, papillary serous adenocarcinoma; IDC, infiltrating ductal carcinoma; TRS, tumor reductive surgery; TAH + BSO, total abdominal hysterectomy + bilateral salpingooophorectomy; MRM,modified radical mastectomy; RM, radical mastectomy; PC, carboplatin + taxol; PAC, carboplatin + adriamycin + cytoxan; CMF, cytoxan + methotrexate+ 5-FU; CAP, Cytoxan + adriamycin + cisplatin

Freshly cultured ovarian TAL recognize FBP peptide E39 after stimulation with DC-E39. Freshly isolated TAL cultured in media containing IL-2 express either low levels of Ag specific cytotoxicity or high non-specific lytic activity during the first 7-10 days of culture. Although the non-specific cytolytic activity decreases over time, it is important to identify approaches to enhance specific CTL activity early and rapidly. This study has focussed on TAL samples which showed either low levels of specific recognition of E39 or E39 recognition was non-specific compared with T2-NP. Representative results for all samples are shown in Figure 1A. To examine the specific recognition of FBP peptides, we cultured isolated OTAL1 in IL-2 without specific stimulation for 1 week. Then the OTAL1 was tested at two different E:T ratios in 5-h chromium release assays for recognition of peptides E39 and E41 presented by T2 cells. As demonstrated in Figure 1A, the results showed preferential recognition of E39 over E41.

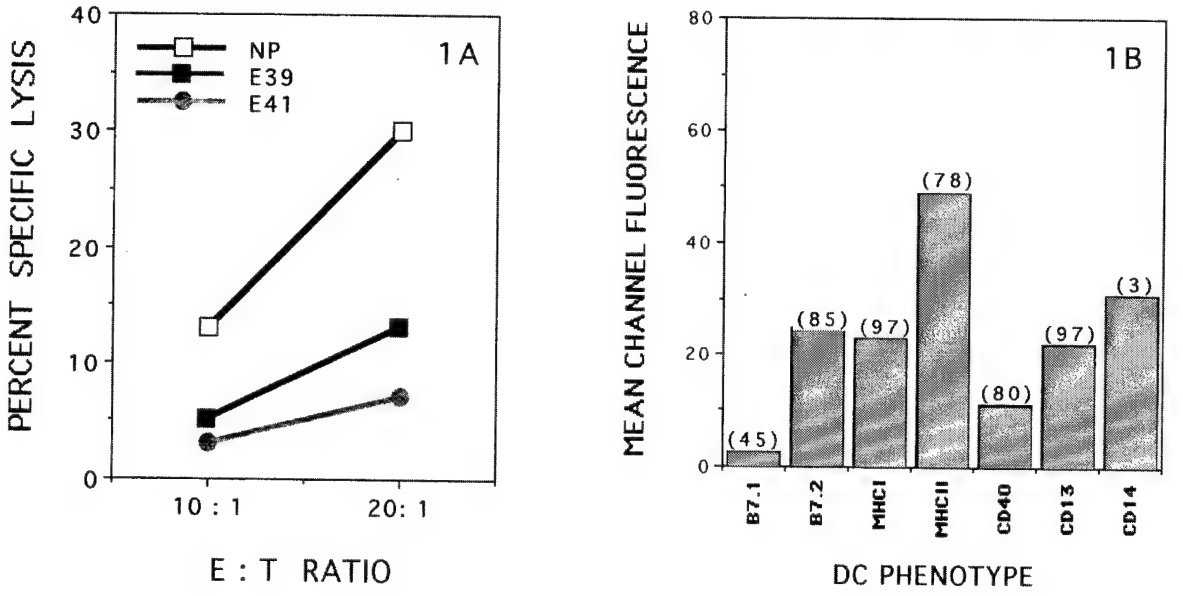


Figure (1A). *Freshly cultured ovarian TAL1 recognize FBP peptide E39. Fresh isolated TAL cultured in IL-2 expressed high levels of non-specific Ag cytotoxicity. The 5-h Cr release assays for the recognition of peptides E39 and E41 cells showed preferential recognition of E39 over E41. Experimental details are presented in the material and methods section. (1B). Cell surface phenotype of DC generated after GM-CSF + IL-4 cultured. PBMC derived DC express high levels of MHC-I, MHC-II and CD86 (B7.2) but low levels of B7.1 (CD80) and CD40. The CD14+ cells were less than 3% of DC, while the CD13+ marker was expressed on more than 97%. Parentheses ( ) indicate the percent positive cells.*

However OTAL1 showed significantly higher recognition of DC-NP compared with DC-E39, suggesting a high percentage of non-specific or cross reactive lytic effectors.

To determine whether the E39 specificity can be induced or enhanced, we used as APC HLA.A2+ matched dendritic cells (DCs) from healthy donors. The phenotype of DC generated after GM-CSF + IL-4 is shown in Figure 1B. They expressed high levels of MHC-I and CD86 (B7.2) but low levels of B7.1 and CD40. The CD14+ cells were less than 3% of DC, while the CD13+ marker was expressed on more than 97% cells. This phenotype is characteristic of immature DC. We pulsed DCs with the peptide E39 and then used DC-E39 to stimulate the OTAL. Since the responders and stimulators were from different individuals which shared only HLA. A2, a certain level of allo-specific and/or cross-reactive specificity was expected. Therefore, in all experiments, the OTAL and BTAL were stimulated in paralled with DC-NP. The parallel stimulations with DC-NP and DC-E39 were done to established the contribution of allospecific responses to the overall increase in lytic activity. Furthermore, if high affinity E39-specific CTL were present and deleted by DC-E39 stimulation, they would have been detected in the DC-NP stimulated cultures.

In most TAL the E39 specificity was induced at the first stimulation. When increased E39 specific recognition was not induced at the first DC-E39 stimulation, it was induced at restimulation. For example, at the first stimulation with DC-E39, the specific lysis of DC-NP vs DC-E39 by OTAL2 were 13.2% vs 15.1% respectively (Figure 2A). When we restimulated the same OTAL2 with DC-E39 again, for 1 more week, we observed a significant increase in DC-E39 recognition compared to DC-NP : 25.7% vs 15.3%, ($p < 0.0002$) (Figure 2B).

Increased CTL-mediated Cytotoxicity of FBP peptide E39 stimulated TAL. E39 (FBP, 191-199) appears to be the immunodominant FBP epitope. CTL assays were performed to determine whether the DC-E39 stimulation increased the levels of recognition of the stimulating antigen. To address this question, we used E39 pulsed T2 as targets. The CTL assays were performed with four ovarian and two breast TAL as effectors at an E:T ratio of 20:1. The results demonstrate that after DC-E39 stimulation all four ovarian TAL and one of two breast TAL showed specific recognition of E39 compared to DC-NP, (Figure 3A). OTAL1 and OTAL3 showed highest specific lysis compared to control, 73.6% and 29.6% respectively, followed by OTAL2 and OTAL4 as 25.7% and 41.8% respectively. These differences were all statistically significant compared to controls, ($p < 0.05$). These results also demonstrate that stimulation with DC-E39 resulted in the highest levels of cytotoxicity against E39 in OTAL but not in BTAL. Control stimulation with DC-NP, representive of allo-stimulation alone did not induce or enhance the specific recognition of E39 (Figure 3B). This is

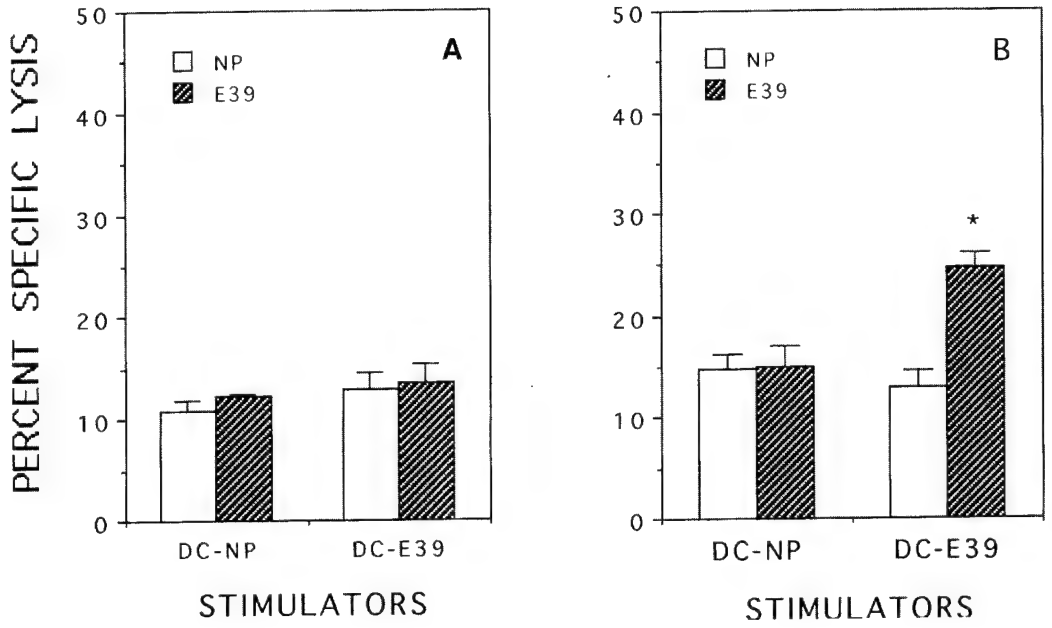


Figure 2(A,B). *Induction of E39 specificity in the ovarian TAL2 require restimulation with DC-E39. Figure (2A), in most TAL the E39 specificity was induced at the first stimulation. OTAL2 required restimulation as shown here. At first stimulation, the specific lysis of OTAL2 stimulated with DC-NP vs DC-E39 were 13.2% vs 15.1% respectively. Figure (2B), when we restimulated OTAL2 with DC-E39 again after one more week, we observed a significant increase in E39 recognition by DC-E39 stimulated OTAL2 compared to DC-NP, 25.7% vs 15.3%. ($p < 0.0002$), (Figure 2B).*

equally true for both TAL where the E39 specificity was present (OTAL1, BTAL2) or abscent. These results together with the results in Figure 3A demonstrate the induction of E39 specific CTL-TAL recognition by Ag stimulation. These results are summarized in Table II. Our results also demonstrate that E39 specific CTL were consistently induced in ovarian TAL but not in the breast TAL.

T Cell receptor Vß expression of TAL stimulated with FBP peptide E39 pulsed DC. We wanted to investgate whether DC-E39 stimulation induced specific changes in the TCR Vß expression. This was achieved by comparing specific TCR Vß expression in DC-E39 versus DC-NP stimulated TAL. To determine the TCR Vß expression in TAL isolated from the ovarian and breast cancer patients, we used monoclonal antibodies (mAb) to stain for 9 different TCR Vß families, Vß3.1, Vß5a, Vß6.7, Vß8a, Vß9, Vß12, Vß13 and Vß23. After 5 days stimulation with DC-E39 and DC-NP, two color FACScan analysis was performed for CD8 and Vß expression. In this part of the study, we investigated the expression of various TCR Vß families in TAL populations from distinct patients stimulated with DC-E39. Complete results for TCR Vß expression by OVTAL1 are shown in Figure 4. Among the 9 different TCR Vß families tested, we found that the expression of levels of only two Vß families was significantly higher (by more than 5% increase) than the one induced by DC-NP. These families were Vß9 and Vß13. The expression of other Vß families (Vß3.1, Vß5a, Vß8a, Vß17 and Vß23)

Table II. *The E39 reactivity CTL response according to TCR Vβ expression on TAL from ovarian and breast cancer patients.*

	Specific E39 reactivity		
	Before	After	Increased
Ovarian TAL			
Patient 1	+	+	↑
Patient 2	-	+	↑
Patient 3	-	+	↑
Patient 4	-	+	↑
Breast TAL			
Patient 1	-	+	↑
Patient 2	+	-	-

showed some increase (by less than 5% Vß expression) than the corresponding Vß expression in the DC-NP stimulated cultures. The expression levels of Vß (6.7 and Vß12 did not changed. Comparative results for six TCR Vß families are shown in Figure 5A.B. The results show significant (> 5%) increase in TCR Vß9, Vß13, and Vß17 in OVTAL stimulated

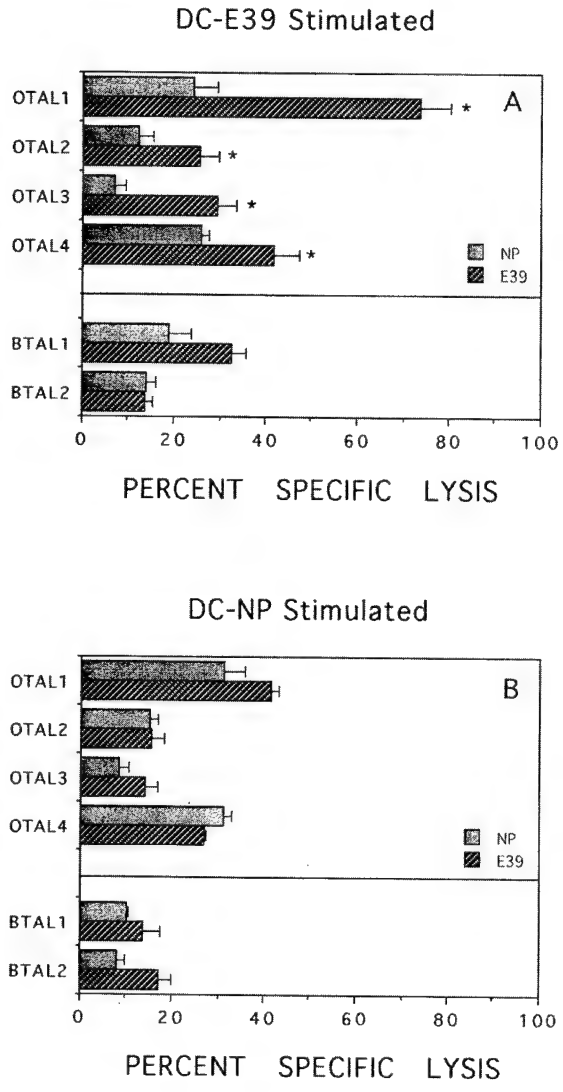


Figure 3. *Increased CTL-mediated recognition of peptide E39 by DC-E39 stimulated TAL. The CTL assays were performed with four ovarian and two breast TAL as effectors at an E:T ratio of 20:1, (Figure 3A). After DC-E39 stimulation all 4 OTAL and 1 of 2 BTAL showed specific recognition of E39 compared to DC-NP. The % specific lysis are OTAL1 73.6%, OTAL2 25.7%, OTAL3 29.6%, OTAL4 41.8% and BTAL1 31.9%. These difference are statistically significant. ($p < 0.05$) Figure 3B. Control stimulation of ovarian and breast TAL with DC-NP did not enhance the specific recognition of E39.*

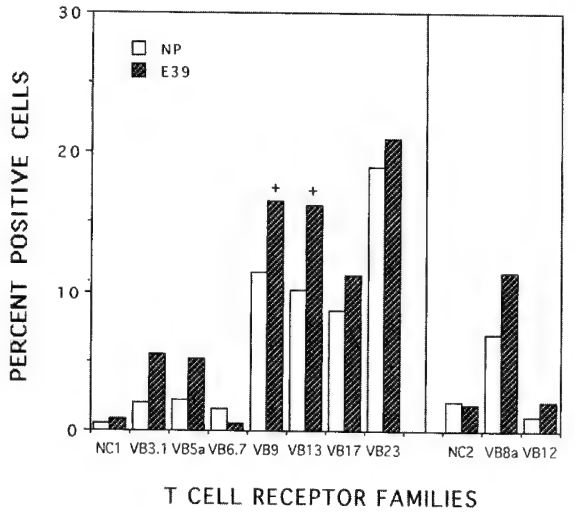


Figure 4. *TCR Vβ expression of OTAL1 stimulated with DC-E39. Among the 9 different TCR V(families tested, we found that the expression of levels of only two Vβ families, Vβ9 and Vβ13 was significantly higher (by more than 5% increase) than the one induced by DC-NP. The expression of other Vβ families, Vβ3.1, Vβ5a, Vβ8a, Vβ17 and Vβ23 showed some increase (by less than 5%) than the corresponding Vβ expression in the DC-NP stimulated culture.*

with DC-E39 (Figure 5B) compared with the same TAL cultured and stimulated by DC-NP (Figure 5A). Since each of these families was expressed at levels which ranged between 4-8%, the increase observed was suggestive that the levels of particular Vß families increased by 80-100%. Therefore, DC-E39 significantly enhanced the TCR Vß expression of certain families suggesting that the increase is due to antigen stimulation.

There were also differences in the expression pattern of TCR Vß families in BTAL compared to OTAL. The Vß17 and Vß 23 families were elevated in the BTAL1 after DC-E39 stimulation compared to control, DC-NP stimulated BTAL1, (Figure 5C). Thus the ovarian and breast TAL stimulated with DC-E39, shared the specific increase in Vß17, but the other dominant familes were different: Vß9 and Vß13 in OTAL versus Vß23 in BTAL. These results suggests that E39 stimulated CTL recognition of the epitope formed by E39-HLA. A2 involves TCR elements which are shared by only certain TCR Vß families but not by others.

The specificity of TAL recognition of the FBP-derived peptide E39. To confirm the involvement of the cells expressing the dominant TCR Vß families, in specific recognition of the HLA-A2/E39 peptide complex on ovarian cancer cell line SKOV3.A2 by CTL, inhibition assays were performed. The anti-TCR Vß antibodies were added at the beginning of incubation of a standard cytotoxicity assay. We chose the OTAL2 as effector in the inhibition assay because of the significant increase in Vß17 (shared with BTAL) and Vß9 (shared with OTAL1). We hypothesized that, if Vß9 and Vß17 TCR on CTL are involved in tumor lysis, adding anti-TCR Vß9 and Vß17 antibodies could competitively inhibit the tumor killing. The results obtained demonstrated successful inhibition of tumor lysis of specific Vß9 and Vß17 mAb. Inhibition of OTAL2 lysis was dependent on the concentration of the anti-TCR Vß antibodies added. The anti Vß9 and Vß17 effectively inhibited 40-50% of the tumor lysis. ($p < 0.0002$) (Figure 6A). In contrast, TCR Vß5 and Vß13

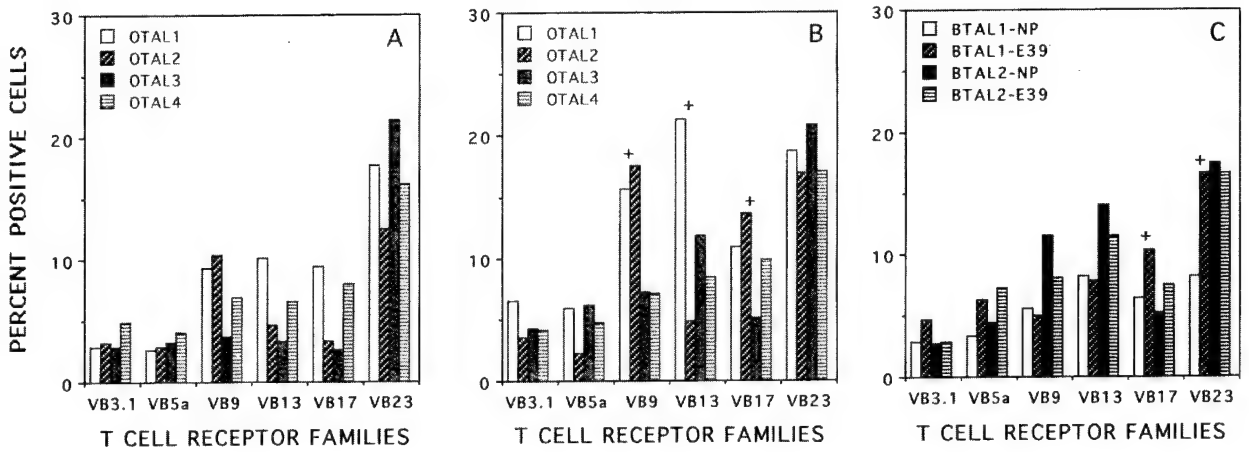


Figure 5. *Comparative expression of six TCR Vβ families by four OTAL (A,B) and two BTAL (C). Significant (> 5%) increase in TCR Vβ9, Vβ13 and Vβ17 in OTAL stimulated with DC-E39 (Figure 5B) compared with the same TAL cultured in the same conditions and stimulated with DC-NP (Figure 5A). There were also differences in the expression pattern of TCR Vβ families in BTAL compared to OTAL. The Vβ17 and V(23 families were elevated in the BTAL1 after DC-E39 stimulation compared to DC-NP stimulation (Figure 5C).*

antibodies did not inhibit CTL lysis. These findings suggest that the CTL specific for this epitope expressing TCR Vß9 and Vß17 antibodies contribute significantly to the recognition of this ovarian cancer cell line.

To confirm that the peptide stimulated TAL recognized an endogenous presented epitope, we performed cold-target inhibition assays using autologous tumor as target, and T2-E39 as inhibitors. The results show that DC-NP stimulated OTAL2 lyse only marginaly autologous tumor. In contrast DC-E39 stimulated OTAL2 exhibited to significantly higher (2-3 fold) levels of lysis ($p < 0.05$). This lysis was inhibited to the levels induced in the DC-NP by T2-E39. These results indicate that DC-E39 stimulated ovarian TAL recognize similar epitope with E39 on their autologous tumor (Figure 6B).

Discussion

In the present study we investigated whether the FBP peptide E39 a novel tumor antigen, can be used as an immunogen for activation of ovarian and breast CTL-TAL. The important function of TAL is to lyse tumor cells. If E39 specific CTL are present in the ovarian TAL, stimulated CTL-TAL can specifically recognize E39 and lyse experimental tumors. However, stimulation and/or restimulation with peptide pulsed DC may also induce apoptosis or silencing of CTL if appropriate cytokines are absent. For this reason each TAL was stimulated in parallel with DC pulsed with or without E39 to determine whether the E39 specificity decreased or increased. As shown in Table II, we found that in five out of six patients E39 stimulation resulted in increased E39 specific CTL reactivity. It was interesting to note that, in all four cases of ovarian TAL there was an increase in E39 specific TAL. These responses were obtained in 3 out of 4 ovarian TAL and 1 out of 2 breast TAL by a first peptide stimulation. Only OTAL2 required a second stimulation with DC pulsed with E39 to elicit E39 specific cytotoxicity. The levels of increase in E39 specificity were higher in OTAL1, OTAL2, and OTAL3 and lower in OTAL4 and BTAL1. For the BTAL2 the levels of E39 recognition in fact decreased, suggesting that both activation and functional silencing are possible.

To induce this E39-specific CTL activity we used as APC, DC from four healthy donors. These donors were HLA.A2 matched with the responders and randomly selected so preferential bias in the favor of inducing one phenotype or another could be ruled out. Of interest, although high levels of allo-specific responses were expected, we found that E39 specificity was higher then the allo-responses.

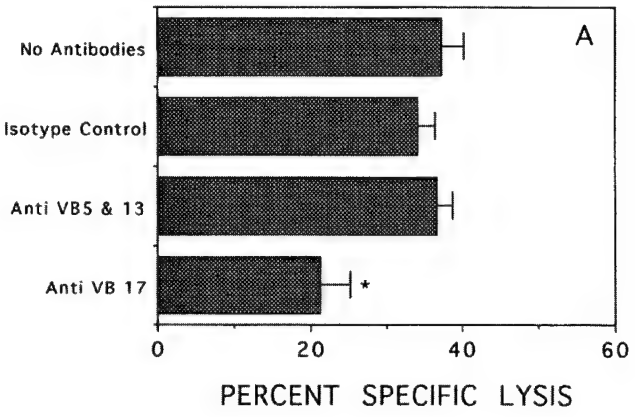
Since the responders and stimulators were only HLA.A2 matched, it was of interest to determine if the increase the specific lysis correlated with the increase in expression of certain Vß families. Increased representation of Vß families would indicate preferential expansion of a clone or group of clones expressing the same TCR family. Furthermore this increase should be paralleled by a decrease in other families since the sum of Vß families should not exceed 100%. For this reason we compared the % Vß expression in E39 and NP stimulated TAL. The results show that, of nine TCR Vß families tested, only three: Vß9, Vß13, and Vß17 preferentially increased after stimulation with DC-E39.

TCR components that contribute to ovarian tumor recognition by CTL have been studied extensively in past several years. Most studies were focused on the phenotype analysis of isolated and cultured ovarian and breast TAL with mixtures of $CD8^+$ and $CD4^+$ cells [10, 30, 31]. A previous study by Fisk et al, analyzed the TCR Vß phenotype

repertoire of $CD3^+$ $CD8^+$ $CD4^-$ CTL reacting with ovarian tumors [28]. They found significant correlation between percentage of TCR Vß 3 and Vß17 family expression and autologous tumor lysis [28]. Peoples and collaborators also demonstrated that the presence of tumor specific CTL in the TAL of ovarian cancer patients. These CTL recognized shared Ags in an HLA-A2 restricted manner. The antitumor activity was mediated by tumor specific CTL of the Vß2, Vß3 and Vß 6 families. Vß 3 and Vß 6 recognized TAA that are derived from the HER2/neu gene and presented in the context of HLA-A2 [32]. The TAL tested in previous studies were not stimulated with Ag. In this study, we reach similar conclusions. After Ag stimulation overexpression of TCR Vß 9 and Vß 17 correlated with the increase in specific tumor lysis.

Many immunological studies are focused on ovarian cancer aiming to idenfify the Ag recognized by TAL because it provides an unique model for the study of the immune response to epithelial cancer. Ovarian cancer has distinct forms of tumor growth pattern. It grows either as single cells in the malignant ascites or as a bulky solid mass. In either case, the tumor specifically induces a T cell response. Tumor Ag identification in ovarian cancer is significant and for other epithelial tumors. These epithelial tumors share common CTL recognized TAA and this feature lead to the development of TAA specific vaccines. Previous studies demonstrated that, the endogenous cellular immune response does exist in a variety of epithelial cancers, and that this response involves the specific recognition of antigenic peptides presented by MHC-I. Currently the established known tumor Ag are MUC-1 and HER2. MUC-1 expression is increased by 10-40 fold in breast cancer compared to normal cells. HER2 has been shown to provide endogenously recognized antigenic peptides but it is overexpressed in about 30% of ovarian and breast cancers. Therefore it is important to find potentially widely applicable CTL recognized Ag for the development of epithelial cancer vaccines.

FBP was identified with a mAb raised against the choriocarcinoma cell line Lu-75c and independently as the Ag recognized by the MOV18 and MOV19 mAb, and the protein that has high affinity to folate [22-25]. Low expression of FBP was observed in some specialized epithelia, such as choroid plexus, lung, thyroid, kidney, and sweat glands. However the highest levels of FBP overexpression were found in ovarian carcinoma. More than 90% of all ovarian carcinomas tested showed elevated levels of this protein. The elevated levels of FBP are as high as 80-90 folds of that of normal tissue [22-25]. Other epithelial tumors, colorectal, breast, lung, and renal cell carcinoma have been also shown to overexpress the LK26/FBP antigen about 20-50% [22-25]. The variety and widely expressed levels of FBP in various epithelial tumors makes an ideal target for the immunotherapy. Inspite of attempts to target FBP antibody conjugates and antifolates the clinical results have been minimal. One reason for the limited efficiency of antibody therapies may be that most antibodies do not recognize FBP in native form, and that FBP has a short extracellular domain which makes the access of antibody/antifolates difficult. As demonstrated in this study, if the FBP derived peptide, E39, can specifically activate ovarian cancer associated CTL then it can be applied to immunotherapeutic strategies. From previous studies, we



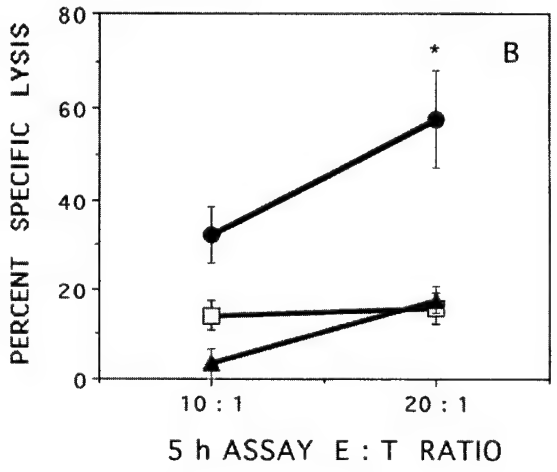


Figure 6A. *DC-E39 stimulated TAL expressing V(17 recognize ovarian tumors. The anti-TCR Vβ antibodies were added at the beginning of incubation of a standard cytotoxicity assay. We choose the OTAL2 as effector in the inhibition assay because of the significant increase in Vβ17 (shared with BTAL). The results obtained demonstrated successful inhibition of tumor lysis by specific anti-V(17 mAb. The anti Vβ17 mAb effectively inhibited 40-50% of the tumor lysis. ($p < 0.0002$). In contrast, TCR Vβ5 and Vβ13 antibodies did not inhibit CTL lysis. Figure (6B): To confirm that the peptide stimulated TAL recognized an endogenous presented epitope, we performed cold-target inhibition assays using autologous tumor as target, and T2-E39 as inhibitors. The results show that DC-NP, stimulated OTAL2 did lyse only marginal autologous tumor, (square in graph). In contrast DC-E39 stimulated OTAL2 exhibited significantly higher (2-3 fold) levels of lysis ($p< 0.05$), (circle in graph). This lysis was inhibited to the levels induced in the DC-NP by T2-E39, (triangle in graph).*

know that adoptive immunotherapy can reduce tumor size in some of solid tumors such as melanoma and renal cell carcinoma [36-37], and it can prolong the survival in advanced ovarian carcinoma when combined with conventional chemotherapy[38]. These results were obtained with TIL/TAL. Using specific CTL-TAL directed toward known tumor epitopes such as FBP derived peptide, E39, we may improve the results appreciably. The FBP appears to be the next candidate for the use as a target for the cellular immunity.

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Received April 14, 1999
Accepted May 3, 1999

[CANCER RESEARCH 57, 87–93, January 1, 1997]

Identification of Naturally Processed Human Ovarian Peptides Recognized by Tumor-associated CD8$^+$ Cytotoxic T Lymphocytes

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ABSTRACT

Identification of naturally processed peptides recognized by tumor-specific CTLs may lead to epitope-specific tumor vaccines. Because these epitopes may be expressed differently on epithelial tumors and may differ in their ability to induce CTL *in vivo*, we have isolated the HLA-A2-peptide complexes by immunoaffinity from an established ovarian tumor line transfected with and expressing *HLA-A2* gene. High-performance liquid chromatography-fractionated peptides were used to reconstitute epitopes recognized on HLA-A2 by three HLA-A2$^+$ CD8$^+$ CTL lines. These lines recognized at least three of the same groups of fractions (designated SKOV3.A, -B, and -C) but showed differences in the pattern of recognition of other fractions. To gain insight in the epitope distribution by freshly isolated ovarian tumors, we compared the recognition of peaks SKOV3.B and -C with the corresponding peaks from an ovarian tumor (OVA-6) that expressed similar levels of HLA-A2, using one of these lines (CTL-OVA-5) as indicator. CTL-OVA-5 recognized a large number of epitopes from peaks B and C rechromatographed on more resolving high-performance liquid chromatography gradients. Although a number of peaks appeared to be coincident on both SKOV3 and OVA-6, an even higher number appeared either not to overlap or to overlap only partially. These findings, which represent the first analysis of the epitopes presented by a patient tumor, suggest that the use of tumor line-derived peptides for vaccination may require selection of the epitopes corresponding to the ones presented by freshly isolated human tumors.

INTRODUCTION

The development of rational immunotherapy approaches for human cancers will depend on a detailed understanding and quantitation of the host antitumor responses. These responses involve recognition by effector CTLs of specific epitopes on malignant tissues and immunogenic epitopes, *i.e.,* peptides that can induce *in vitro* and *in vivo* antitumor responses. CTL epitopes are short peptides (8–10 amino acids long) that are presented on the cell surface by MHC class I molecules (1). Because peptides of distinct sequences can be distinguished by their physicochemical characteristics (2), elution under defined pH and concentration conditions proffers an approach to map the universe of epitopes recognized by tumor-reactive CTLs. This may allow us to establish which peptides (or groups of peptides) correspond to common dominant epitopes recognized by *ex vivo*-induced tumor-reactive CTLs. Due to T-cell receptor plasticity, the common epitopes can be reconstituted by either unique or cross-reactive peptides (3, 4). Furthermore, for CTL induction, tumor Ags[2] may be presented to T cells by professional Ag-presenting cells (5), which can process particulate Ags for MHC class I presentation (6, 7). Thus, identification of the natural peptides presented in association with MHC molecules on human tumors should be important in the development of novel approaches for tumor-specific CTL induction.

To date, with the few exceptions of melanoma Ag, gp100, and MART-1 (8–10), there is limited information on the identity and density of antigenic peptides and CTL epitopes presented by human solid tumors. The presence, distribution, and density of these epitopes on freshly isolated epithelial tumors is still unknown. Our studies as well as research from other laboratories have shown that multiple epitopes can be recognized on epithelial tumors (*i.e.,* ovarian, colon, pancreatic, breast, and lung; Refs. 11–13). Because acid-eluted peptides have been shown to be immunogenic by inducing antitumor immunity to established model tumors (14, 15), this raises the need for adequate sources of tumor epitopes for human studies. This can be accomplished with freshly isolated primary tumors only rarely because the small amount of peptides recovered preclude peptide sequence analysis. An approach to overcome these limitations is to use ovarian and breast tumor lines expressing the MHC class I molecule of interest to address the question of whether the patterns of epitope recognition by tumor-reactive CTL on the lines and freshly isolated human tumor are similar.

To address these questions, we have investigated the identity of epitopes presented by HLA-A2 on tumor targets recognized by HLA-A2$^+$ CD8$^+$ CD4$^-$ CTL lines isolated from patients with ovarian cancer. In this report, we characterized the pattern of CTL epitopes extracted from a freshly isolated ovarian tumor and HPLC separated by two consecutive gradients of ACN, and we identified common and distinct epitopes between this tumor and HLA-A2-bound peptides fractionated from an established ovarian tumor line, SKOV3. Although recognition of a number of epitopes defined as bioactive peaks eluting with distinct retention times appeared to correlate with the cell number used and the levels of HLA-A2, on these tumors for some common epitopes such a correlation was not observed, suggesting that MHC class I expression is not the only determining factor in tumor Ag expression.

MATERIALS AND METHODS

Tumor Cells and Cell Lines. The following human tumor lines were used in these experiments: (*a*) ovarian tumor line SKOV3 (HLA-A3, A28, B18, B35, and Cw5) stably transfected with the gene for HLA-A2 (16). The gene for HLA-A2 was provided kindly by Dr. William E. Biddison (National Institute of Allergy and Infectious Diseases, NIH). A tumor clone, SKOV3.A2.1E4, expressing high levels of both HER-2 and HLA-A2, has been designated as 1E4 and was selected for expansion in large numbers and peptide fractionation experiments; and (*b*) C1R:A2 cells (a kind gift from Dr. Biddison), which express only HLA-A2 on the surface, stably transfected in our laboratory with the gene encoding for the *HER-2* proto-oncogene (plasmid pCMV.HER-2 encoding a full-length HER-2.cDNA). This plasmid was a kind gift of Dr. Mien-Chie Hung (Department of Tumor Biology, M. D. Anderson Cancer Center). C1R.A2 cells were cotransfected with the plasmid SV2.Hygro (American Type Culture Collection). HLA-A2-transfected SKOV3 and C1R:A2 cells were selected with 250 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD). C1R.A2.HER-2 cells were also selected with 50 μg/ml of hygromycin B in addition to G418. This concentration was found to result in the death of more than 50% of untransfected C1R:A2 cells within 4 days in parallel

Received 9/9/96; accepted 11/1/96.

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[2] The abbreviations used are: Ag, antigen; mAb, monoclonal antibody; MCF, mean channel fluorescence; HPLC, high-pressure liquid chromatography; ACN, acetonitrile; TFA, trifluoroacetic acid; Rt, retention time; TAL, tumor-associated lymphocyte; IL, interleukin; EGF-R, epidermal growth factor receptor.

experiments. After selection with hygromycin B, C1R.A2.HER-2 cells were cloned by stringent limiting dilution. At least 20 C1R.A2.HER-2 clones (designated HER-2.A-T) were isolated, expressing variable levels of HER-2 receptor on their surface. A clone of C1R:A2.HER-2 cells designated here as HER-2.J was selected for additional studies.

Ovarian tumor cells were collected from the ascitic fluid of a patient with epithelial ovarian cancer and separated from debris and lymphocytes by centrifugation over two gradients of Ficoll as we have described previously (17, 18). They were HLA-A2$^+$ and expressed high levels of HER-2 (HER-2^{hi}). These tumor cells were designated as OVA-6.

Effector CTL Lines. T-cell lines from TALs were grown in RPMI 1640 medium containing 10% FCS and supplemented with 2 mM L-glutamine, and 100 μg/ml gentamycin (complete RPMI medium) and 50–100 units/ml of IL-2 (Cetus). Most effectors expanded, because T-cell lines contained >95% CD3$^+$ cells and variable proportions of CD8$^+$ and CD4$^+$ cells. For these experiments, CD8$^+$ cells were selected on mAb-coated plates as we described (16). The resulting cells were >95% CD3$^+$ CD8$^+$ cells. Effector CTL-3 used in these studies has been reported previously (16). CTL-4 and CTL-5 were also obtained from TALs from other patients with adenocarcinoma of the ovary. The corresponding tumors were HLA-A2$^+$ and overexpressed HER-2 (HER-2^{hi}).

Immunofluorescence. Target cells were tested in fluorescence experiments to confirm the expression of HLA-A2, MHC class I, and HER-2, as described previously (15). Hybridomas secreting mAbs BB7.2, MA2.1, and GAR-3 (HLA-A3 specific) were obtained from American Type Culture Collection. In brief, OVA-6, SKOV3, and HER-2.J cells were incubated with mAb specific for MHC class I and mAb *Ab2* specific for the extracellular domain of HER-2 (Oncogene Science) followed by FITC-conjugated goat antimouse IgG. Surface Ag expression was determined by fluorescence-activated cell sorting using a FACScan (Becton Dickinson) with a log amplifier. CD3, CD4, and CD8 Ag expression on the effectors was determined by immunofluorescence with corresponding mAb. C1R.A2 clones were designated as HER-2^{hi} when MCF for HER-2 expression was ≥40. Expression of CD18, CD11a (leukotactic factor activity 1), CD54 (intercellular adhesion molecule 1), and CD58 (leukotactic factor activity 3) was also tested the corresponding antibodies (Becton Dickinson).

Tumor Peptide Extraction. SKOV3.A2.1E4 cells were grown in 10-chamber cell factories (Nunc, Thousand Oaks, CA) in complete RPMI medium. Between 1.0 and 1.5 × 10^9 cells were obtained from one cell factory. For these studies, at least 10^{10} cells of the SKOV3.A2.1E4 cloned line were grown in batches of 1.5–2.0 × 10^9 cells. Cells were collected and washed three times with cold PBS. Furthermore, cells were lysed using the buffer described previously by Slingluff *et al.* (19) containing protease inhibitors (aprotinin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride, and iodacetamide), in PBS, with the difference that 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfate 0.5% was used as a lysing agent to minimize binding to C18 columns.[3] This solution is designated here as lysis buffer. $MgCl_2$ at 6 mM and glycerol at a 20% final concentration were included in the lysis buffer to minimize the denaturation of extracted proteins. Detergent-solubilized extracts of SKOV3.A2 and OVA-6 cells were obtained after centrifugation at 40,000 × *g* for 2 h. HLA-A2.1 was isolated from the supernatant obtained from SKOV3.A2 cells by affinity chromatography on protein A-Sepharose prebound with mAb BB7.2 as described (19), except that the cell extracts were preabsorbed on protein-A Sepharose (Sigma Chemical Co., St. Louis, MO) to minimize nonspecific binding. The affinity column was washed with PBS containing 0.25 M NaCl with monitoring of the absorbance ($A_{210\,nm}$) (for peptide bond) and then eluted with 0.2 M acetic acid in 1.0-ml fractions. Fractions containing material absorbing at 210 nm were boiled for 5 min to allow dissociation of peptides from HLA-A2, centrifuged through an Ultrafree CL unit (3-kDa cutoff) (Millipore), and lyophilized. Peptides with masses <3 kDa were pooled, lyophilized, and separated by reverse-phase HPLC. This approach yields primarily HLA-A2-bound peptides and to a much lesser extent peptides associated with other MHC class I molecules.

Because freshly isolated ovarian tumors are unique specimens and the corresponding MHC-class I associated peptides are important for identification of epitopes presented by other HLA molecules, peptides were extracted from OVA-6 centrifuged lysates with 0.1% TFA following the approaches of Tsomides *et al.* (20) and Sykulev *et al.* (21). This approach has the advantage of eluting peptides with affinity high for MHC class I. Peptides of mass of 3 kDa or less were isolated by centrifugation through filters with a cutoff of 3 kDa, lyophilized, and separated by HPLC.

Fractionation of HLA-A2-bound Peptides. Tumor peptides extracted from HLA-A2.1 molecules of SKOV3.A2 cells were separated in the first dimension on a Brownlee C18 Aquapore column of 2.1 × 30 mm [pore size, 300 Å; particle size, 7 μm (Applied Biosystems, Perkin-Elmer Corp.)] and eluted with a 60-min gradient of 0–60% (v/v) ACN (Sigma Chemical Company, St. Louis, MO) in 0.1% TFA (Sigma Chemical Company, St. Louis, MO) at a flow rate of 200 μl/min using HPLC system model 1090 (Applied Biosystems). HER-2 peptides E75 (369–377), C85 (971–979), E90 (789–797), and E89 (851–859) identified in the previous studies to be recognized by ovarian CTL-3 line were separated under the same conditions. Their Rts were determined, and their resolution by these HPLC conditions was established.

For the second-dimension HPLC separation, fractions from the first dimension, including the peaks of CTL activity, were pooled in two groups, B (fractions 27–33) and C (fractions 37–45). Each group was lyophilized and reconstituted in the elution buffer corresponding to the gradient used in the second dimension (described below). Then, peptides were injected into a Brownlee C18 Aquapore column of 2.1 × 220 mm, (300 Å; 7 μm) and eluted with shallower ACN gradients. The flow rate was 200 μl/min, and fractions were collected at 1-min intervals. Peak B (fractions 27–33) were separated with a gradient of 0.1% TFA in H_2O for 0–5 min, followed by 0.1% TFA in 0–20% ACN (2%/min) for 6–15 min, 0.1% TFA in 20–40% ACN (0.5% increment/min) for 16–55 min, and 0.1% TFA in 40% ACN (designated as gradient II) for 56–60 min. Peak C fractions (Fxns 37–45) were separated in a linear gradient of 0–60 min of 30–50% ACN in 0.1% TFA thus, with an increment of ACN concentration of 0.3%/min (designated as gradient III). These gradients were selected to allow better resolution of peptides eluting in the corresponding concentration of ACN in the first dimension. For example, peptides eluting in the first dimension between 37 and 45% ACN over 8 min were separated with a gradient of 30–50% ACN over 60 min. Peptides extracted from the freshly isolated tumor OVA-6 were separated by two rounds of HPLC under identical conditions as SKOV3 peptides to allow comparison of biological activity.

Ags. Synthetic HER-2 peptides E75, G89, C85, E90, E91, E92, and the EGF-R peptide F49 (22) were prepared by the Synthetic Antigen Laboratory at the M. D. Anderson Cancer Center using a solid-phase method and purified by HPLC as described (23). The identity of peptides was established by amino acid analysis. The purity of peptides was more than 97%.

CTL Epitope Reconstitution. Reconstitution of CTL epitopes was performed using HPLC fractions from the first and second HPLC dimensions. Fifty-μl aliquots of each fraction were concentrated by vacuum centrifugation (Speed Vac) to approximately 1/10 of its original volume to remove TFA and ACN, reconstituted with water, reconcentrated, and then reconstituted with RPMI medium to the initial volume and added to ^{51}Cr-labeled T2 cells in V-bottomed microtiter plates. After preincubation with peptides for 90 min, effectors were added at 10:1–20:1 E:T ratios, and a standard CTL assay was performed for 5 h as described (15). Control wells were made with T2 cells incubated with equal volumes of the same fraction of HPLC-separated peptides without CTLs, to account for direct cytotoxicity of HPLC fractions themselves.

The percentage of specific lysis was determined from the equation $A - B/C - B \times 100$, where A is the ^{51}Cr release from T2 cells by effectors in the presence of a peptide fraction, B is the release from T2 cells in the presence of the same volume of the same HPLC fraction but in the absence of effectors, and C is the maximum ^{51}Cr release. At least two determinations were made from each fraction.

RESULTS

Similar Patterns of HLA-A2-associated Recognition for Peptides Isolated from an Established Line and a Freshly Isolated Ovarian Tumor. To evaluate the role of the transfected HLA-A2 as a presenting molecule for the ovarian CD8$^+$ CTL, we tested recognition by the ovarian CTL lines CTL-OVA-3 and CTL-OVA-5 of the SKOV3, SKOV3.A2 (1E4), C1R.A2, and C1R.A2.HER-2.J cells in

[3] V. Engelhard, personal communication.

parallel with OVA-6. SKOV3 and SKOV3.A2 cells are of identical origin. Introduction of the *HLA-A2* gene is expected to allow presentation of a number of endogenously processed peptides. Similarly, C1R.A2 and C1R.A2.HER-2.J cells are of identical origin. C1R.A2 cells express a complete and functional antigen-processing and transport system. In these cells, introduction of the *HER-2* gene is expected to lead to the presentation of a number of peptides derived either from HER-2 or from proteins subject to upregulation or stabilization of expression by HER-2 overexpression (24). Because these CTL lines were shown previously to recognize HER-2 peptides (16), failure of the effectors to lyse C1R.A2.HER-2 cells would suggest that peptides recognized are not presented, and/or other peptides derived from HER-2 (which are not recognized) are processed and presented by the C1R.A2.HER-2 cells.

The results in Fig. 1, *A* and *B*, show that target recognition by CTL-OVA-3 and CTL-OVA-5 is associated with HLA-A2 expression. Similarly, both CTL-OVA-3 and CTL-OVA-5 recognized OVA-6 (Fig. 1). Recognition of CTL-OVA-3 and CTL-OVA-5 was inhibited by antibodies to MHC class I but not to MHC class II (data not shown).

For comparative analysis of epitope recognition, peptides were extracted from a freshly isolated ovarian tumor, OVA-6, and the clone SKOV3.A2.1E4 (designated SKOV3.A2). Both tumors expressed similar levels of HLA-A2 (SKOV3.A2; 80% HLA-A2$^+$ cells; MCF, 144; OVA-6: 75% HLA-A2$^+$ cells; MCF, 175). The levels of HER-2 were significantly higher on SKOV3.A2 cells (MCF, 324) compared with OVA-6 (MCF, 160).

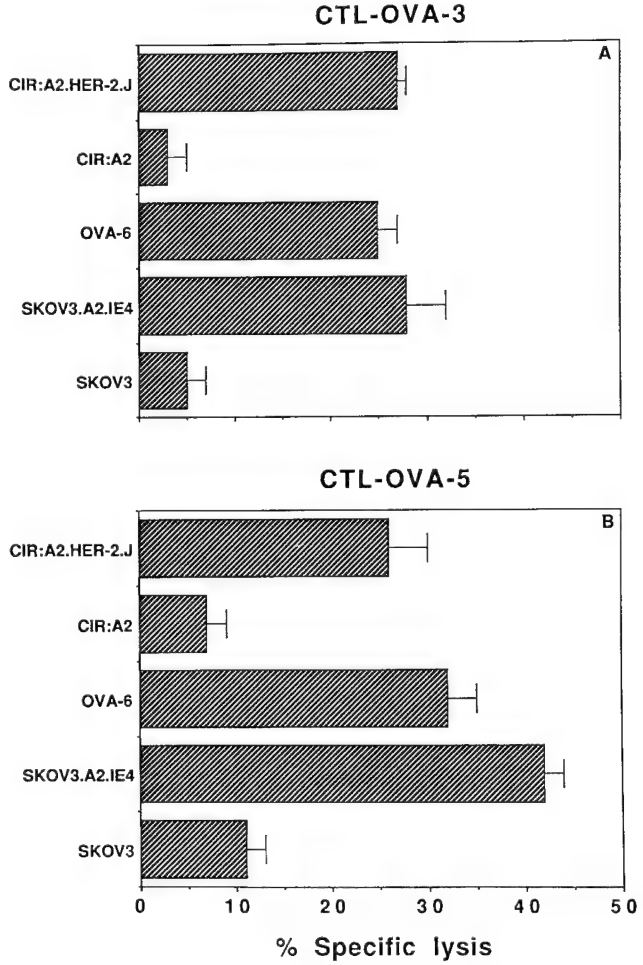

Fig. 1. Recognition by ovarian CD8$^+$ CD4$^-$ CTL lines CTL-OVA-3 and CTL-OVA-5 of the ovarian tumors SKOV3 and OVA-6 and C1R.A2 cells expressing transfected *HLA-A2* and *HER-2* genes. E:T ratio was 20:1. Results were obtained in a 5-h CTL assay.

To establish whether HLA-A2-bound bioactive peptides extracted from SKOV3 can be detected, we compared the recognition of SKOV3.A2 peptides by two ovarian CTL lines, CTL-OVA-3 and CTL-OVA-4 (Fig. 2 *A* and *B*). Equal amounts of SKOV3 peptides separated in the first-dimension HPLC were used for both CTL lines. Analysis of recognition of SKOV3.A2, fractions 10–34, by each of the CTL lines revealed four major peaks of biological activity. Two of three peaks, SKOV3 (fractions 22–27) and SKOV3 (fractions 29–32) appeared to coincide, and the third peak SKOV3 (fractions 18–21) appeared to overlap partially. The positions and heights of the peaks of more hydrophilic peptides eluting with smaller Rts were different between CTL-OVA-3 and CTL-OVA-4. This may suggest that different antigenic specificities are present in each CTL population. Furthermore, differences in the magnitude of response of CTL-OVA-3 and CTL-OVA-4 to peptides in peaks SKOV3 (fractions 22–27) and SKOV3 (fractions 29–32) were observed, suggesting not only the presence of common tumor Ag in the SKOV3 cells, but also differences in the frequency of CTL clones reacting with these peptides. The range of CTL activity observed was similar to the levels reported in recent studies for melanoma (10), and ovarian carcinoma (13).

The ascites that were the source of OVA-6 cells contained mainly tumor cells and very few lymphocytes. These lymphocytes could not be grown in sufficient numbers to perform the CTL assays. The use of high concentrations of IL-2 to promote growth lead to MHC-unrestricted lysis (data not shown). Thus, the analysis of OVA-6 peptides used established CTL lines.

Because of the limited amount of fresh tumor material, identification of bioactive peaks was made only with CTL-OVA-5. For epitope reconstitution studies, we used peptides extracted from different numbers of SKOV3.A2 and OVA-6 cells. The rationale of this approach is that if an epitope is expressed at similar densities in both tumors as shown by Griem *et al.*, the use of different amounts of peptides for epitope reconstitution would be detected as a proportional change in the CTL activity (26). SKOV3 peptides from 9.5×10^7-cell equivalents were added in each well in the CTL assay. For OVA-6, the equivalent number was 5.5×10^7 cells, resulting in a ratio of 1.8:1.0 between SKOV3 and OVA-6 peptides. This ratio was also maintained for determination of recognition of peptides separated in the second dimension. Indeed, the cytotoxicity levels detected with CTL-OVA-5 were in most instances higher with SKOV3 than with OVA-6 peptides (Figs. 2 and 3), suggesting that the epitopes detected using cell lines are the most stimulatory ones.

This should be important for peptide quantitation and epitope identification studies, because differences in CTL epitope expression have been correlated in some (26, 27) but not in other studies (3) with different levels of MHC class I expression. To determine whether a similar pattern of bioactive peptides is expressed by the freshly isolated ovarian tumor OVA-6, recognition of OVA-6 peptides separated in the first-dimension HPLC and of SKOV3.A2 peptides was tested in parallel using as effectors CTL-OVA-5 cells.

We focused on fractions with higher retention times in OVA-6, *i.e.*, the elution positions associated with a potential common tumor Ag shown in Fig. 2, and assayed every fraction in this region to optimize resolution of bioactive peaks. The results in Fig. 2, *C* and *D*, show that a broad peak of SKOV3 (fractions 20–25) corresponds to a double peak of OVA-6 (fractions 22–26); a broad peak of SKOV3 (fractions 28–34) corresponds to two resolved peaks of OVA-6 (fractions 28–29) and OVA-6 (fractions 31–32). A third broad peak of SKOV3 (fractions 39–44) corresponds to peak OVA-6 (fractions 38–41). These results indicate that CTL-OVA-5 recognizes peptides present in

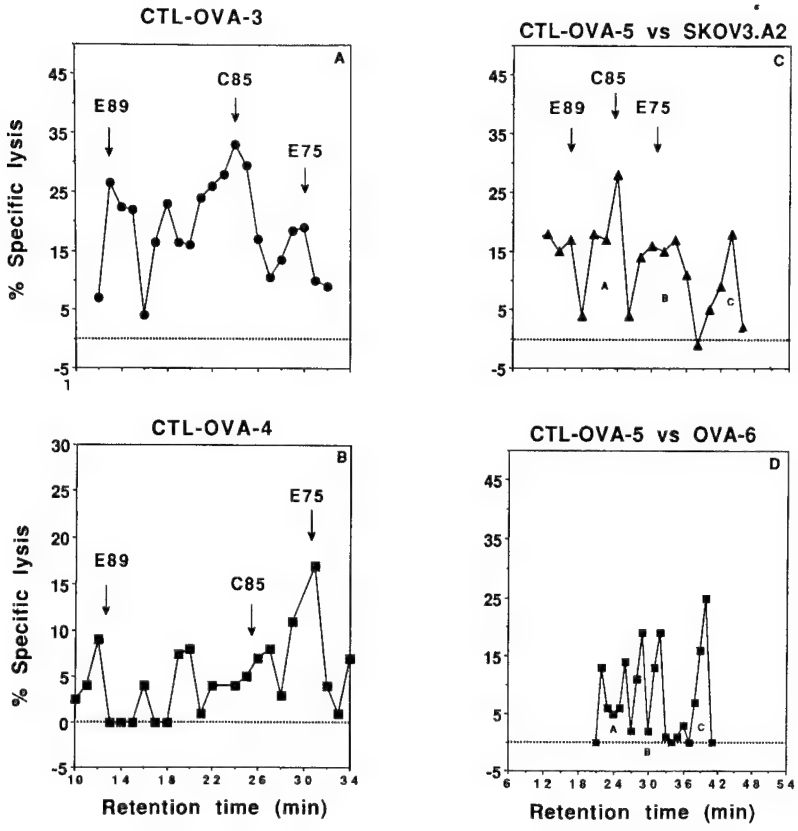

Fig. 2. *A* and *B*, recognition by ovarian CTL lines CTL-OVA-3 (*A*) and CTL-OVA-4 (*B*) of HLA-A2-bound peptides from the SKOV3.A2 cell line fractionated in the first-dimension HPLC. *C* and *D*, recognition by ovarian CTL line CTL-OVA-5 of HLA-A2-bound peptides from SKOV3.A2 cells (*C*) and of TFA-extracted peptides from the freshly isolated ovarian tumor OVA-6 (*D*). Both fractionations employed first-dimension HPLC conditions as described in "Materials and Methods." The E:T ratio was 20:1. Percentages of specific lysis of T2 cells that were not incubated with HPLC fractions were 2% for CTL-OVA-3 and 4% for CTL-OVA-4. These values were not subtracted from the cytotoxicity values induced by peptide fractions, because the control T2 cells were not incubated with column fractions. The cpms of ^{51}Cr release by T2 cells incubated with HPLC fractions in the absence of effectors were in most instances lower than those of ^{51}Cr release of T2 incubated without HPLC fractions. Details are presented in "Materials and Methods." The Rts of the HER-2 peptides separated under the same conditions were: C85 (HER-2, 971–979), 23.7 min; E89 (HER-2, 851–859), 13.4 min; E75 (HER-2, 369–377), 31.5 min; and control hydrophobic signal peptide E91 (HER-2, 5–13), 38.4 min.

overlapping peaks of activity with CTL-OVA-3 and CTL-OVA-4. These peaks are designated as *A*, *B*, and *C* in Fig. 2, *C* and *D*. Furthermore, identification of activity in OVA-6 peptide peaks eluting in the same positions with peaks A–C with a tolerance of ±1 min (1% ACN difference) suggests the possibility that a number of active tumor peptides may be common between the freshly isolated tumor OVA-6 and the SKOV3.A2 tumor line.

Multiple Epitopes Recognized by CTLs Are Presented by HLA-A2 on Ovarian Tumors. Results presented above show that a number of bioactive peaks corresponding to tumor peptides are recognized by ovarian CTL lines. To address the question of whether the epitope repertoire is composed of a limited number of peptides, fractions corresponding to bioactive peaks A–C from SKOV3.A2 and OVA-6 were subjected to an additional round of HPLC separation. To improve resolution, a longer HPLC column (of 220 mm length) of the same diameter and pore size as the one used in the first dimension was employed. Fractions separated in the first-dimension HPLC usually contain a large number of peptides (25). Because not every peptide is active, it is possible that a number of inactive peptides of higher affinity for HLA-A2 compete with the active peptide; thus, the observed CTL activity can appear lower than the activity of the fractions subjected to additional rounds of purification (25). The analysis of CTL activity presented here was focused primarily on the peptides of higher Rt eluting in peaks B and C. To afford comparisons, the CTL-OVA-5 was used as an indicator in all the assays.

With regard to peak B, which eluted as a broad peak of peptides from SKOV3.A2, and a double peak from OVA-6 (Fig. 2, *C* and *D*), fractions 27–33, were pooled, lyophilized, fractionated using a shallower ACN gradient with increments of 0.5% per min, and subjected to CTL analysis. We focused on fractions 30–47, because they eluted at ACN concentrations ranging between 27 and 35%, *i.e.*, the range in which peak B eluted during fractionation in the first dimension. The results are shown in Fig. 3 *A* and *B*. SKOV3 peptides eluted in three major peaks of activity are designated as follows: 1 (fractions 31–36); 2 (fractions 38–42); and 3 (fractions 43–47). OVA-6 peptides eluted in three broad peaks and designated as follows: 1 (fractions 35–40); 2 (fractions 40–43); and 3 (fractions 43–47). They were preceded by a small peak (fractions 32–35) that overlapped with SKOV3, peak B1. Peak B3 was almost coincident in SKOV3.A2 and OVA-6. The peak CTL values and the shape of peaks B1 and B2 of CTL activity in OVA-6 were different from SKOV3. This suggests the possibility that peptides in peaks B1 and B2 differ quantitatively and/or qualitatively in SKOV3 and OVA-6. The fact that peptides in peak SKOV3.B1 are more stimulatory than OVA-6 peptides eluting in the same positions suggests the possibility that these peptides may have been presented and were immunogenic in an earlier-stage tumor.

Peak C, which eluted as a single peak in both OVA-6 and SKOV3.A2 fractions and likely contains more hydrophobic peptides than peaks A and B, was fractionated using a shallower (0.3% increments/min) and thus more resolving ACN gradient. The results are shown in Fig. 3 *C* and *D*. Under these separation conditions, peak C resolved in at least 8 distinct peaks of both SKOV3 and OVA-6 peptides. Comparison of the plotted CTL activity over Rt shows that peaks 2 (fractions 16–19) and 3 (fractions 20–23) are almost coincident. These peaks eluted at ACN concentrations ranging between 35.3 and 37.7%. In addition peak 4 is maximal at 24 min with a broad shoulder at 25–27 min (38% ACN) but is poorly separated (or recovered) in SKOV3. This suggests that several peptides with very similar retention time endowed with biological activity are present in both OVA-6 and SKOV3.A2 cells.

The shape and peak values of the other peptides, or groups of peptides corresponding to peaks 1, 5, 6, 7, and 8, show differences between OVA-6 and SKOV3.A2. SKOV3.C.1 (fractions 13–14) and OVA-6.C.1 (fractions 11–14) elute at similar positions, but the maximal value of SKOV3.C.1 corresponds to the shoulder of peak OVA-6.C1. Similarly, SKOV3.C5 (fractions 25–29) and OVA-6.C5 (frac-

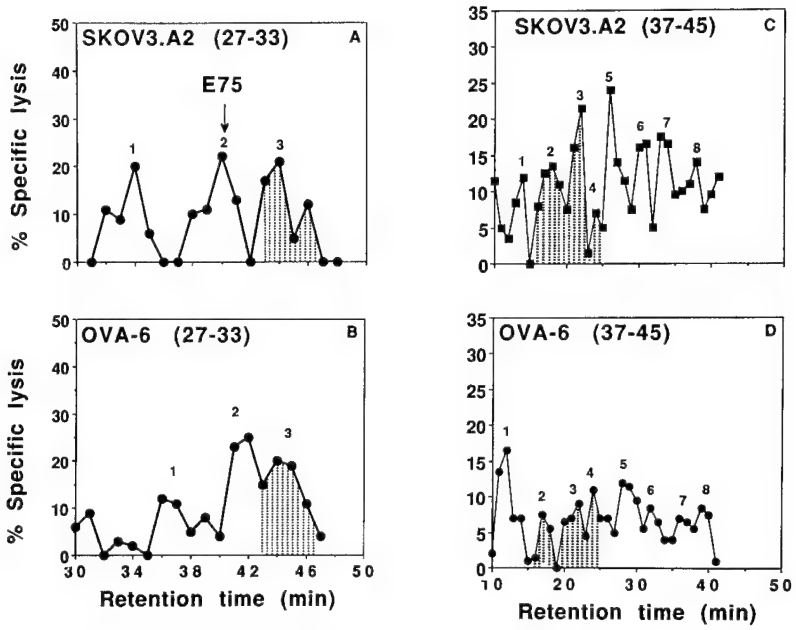

Fig. 3. Recognition by CTL-OVA-5 of second-dimension HPLC fractions corresponding to the B peaks of activity in the first dimension shown in Fig. 2. *A* and *C*, SKOV3.A2 peptides; *B* and *D*, OVA-6 peptides. *Dotted areas* indicate coincident peaks in the B3 peak of activity. The positions of elution of the HER-2 peptides used as markers for these HPLC conditions were: E90 (HER-2, 789–797), 25.4 min; E92 (HER-2, 650–658), 32.4 min; and E75, 40.5 min. The Rt of an EGF-R peptide, F49 (EGF-R, 356–364) KILGNLDFL, was 38.5 min (underlined residues indicate mutations in the E75 sequence). Lysis of T2 cells incubated with CTL-5 was as follows: no peptide (3 ± 1%); E75, 1 μg/ml (14 ± 2%); and F49, 5 μg/ml (2 ± 1%). In the same experiment, lysis of T2 cells by TAL-OVA-6 (autologous with OVA-6) was: no peptide (71 ± 10%); and E75, 1 μg/ml (73 ± 7%). TAL-OVA-6 showed slow growth. The use of high concentrations of IL-2 to enhance proliferation lead to high nonspecific cytotoxicity levels. *C* and *D*, recognition by CTL-OVA-5 of second-dimension HPLC fractions corresponding to peak C of activity in the first dimension shown in Fig. 2, *C* and *D*. *Dotted areas* indicate coincident peaks C2, C3, and C4. There is no lysis by peptide only if compared with control T2 cells, because the cpms released in this group were the same or lower than spontaneous release by T2 cells. The elution positions of the HER-2 peptides used as markers to verify the capacity of separation in gradient III were: F57 (HER-2, 435–443), 14.7 min; E90, 16.1 min; F49, 18.5 min; E75, 19.9 min; and E91, 29.7 min.

tions 28–30) overlap partially. On the basis of comparison of cytotoxicity values, peptides isolated from ovarian cell line appear to be more stimulatory then the peptides isolated from the fresh tumor. The reasons for the observed differences are currently under investigation. First, it should be noted that under these gradient conditions, ±1-min differences in the Rts of peaks of elution reflect differences of only ±0.3% in ACN concentration. Secondly, the amount of HLA-A2-bound peptides isolated from SKOV3.A2 and OVA-6 cells was different because of different amounts of starting material. Although we isolated a large number of OVA-6 cells, the number of OVA-6 cells was significantly lower than the number of SKOV3.A2 cells used in these studies.

To gain insight into the relationship between HLA-A2 expression and epitope density, we compared the cytotoxicity values in the bioactive peaks of SKOV3.A2 and OVA-6 that showed the highest levels of coincidence in their Rts (±1.0 min tolerance). The results are presented in Table 1. These results show that for peaks C2 and C3, the activity in the SKOV3 fractions was significantly higher than in the corresponding fractions from OVA-6. Particularly for the peak of SKOV3.C2, the activity was higher than the one expected from an 1.8:1.0 ratio (tumor line: fresh tumor). The same pattern was observed for the peaks of SKOV3.B1 and the partially overlapping peaks B2, C5, C6, and C7. For peak C4, the CTL activity was significantly higher in the OVA-6 fractions. Similarly, the levels of CTL activity in OVA-6 fractions were the same or higher in peak B3 than the levels observed with SKOV3.A2 fractions. This suggests that if for a number of tumor epitopes the density may correlate with the cell number (and with the levels of HLA expression), for others this does not apply, suggesting that they may be preferentially processed and presented by tumor cells.

Table 1 *Common epitopes on SKOV3.A2 and OVA-6 recognized by CTL-OVA-5*[a]

Peak designation (fraction No.)	Peptides							
	SKOV3.A2				OVA-6			
B3 (43–46)	18	21	5	12	16	20	19	12
C2 (16–19)	8	13	14	12	2	8	6	0
C3 (21–23)	16	22	1		8	9	4	
C4 (24–25)	6	5			17	7		

[a] Numbers indicate the percentage of specific lysis by CTL-OVA-5 of T2 cells incubated with equal volumes of the same HPLC fractions as described (26).

DISCUSSION

In this report, we present evidence that peptides extracted from an established ovarian tumor line and from a freshly isolated ovarian tumor can reconstitute the lytic activity of ovarian CTL isolated from malignant ascites of patients with ovarian cancer. Both ovarian tumors and the effectors shared HLA-A2. Assessment of the number of epitopes recognized suggests that OVA-6 and SKOV3 can present at least 11 distinct epitopes to an ovarian CTL line. Of these 11 epitopes, 4 [1 separated in gradient II (B3) and 3 separated in gradient III (C2, C3, and C4)] appear to be shared between SKOV3 and OVA-6, using as comparison factors the Rts and the shapes of the peaks. The number of shared epitopes is likely to be higher if at least some of the peptides present in the partially overlapping peaks B2, C1, C5, and C6 are identical in both samples. The Rts of synthetic peptides used as markers (Figs. 2 and 3) show that both gradients II and III are quite resolving because they can separate the HER-2 peptide, E75 (369–377), from the corresponding mutated EGF-R (356–364) peptide. Similarly, mass-spectrometric analysis of ions presumed to be peptides in peaks of gradient II and III show that each ion was present

mainly in two and no more than three consecutive HPLC fractions.[4] Thus, it is likely that most of the nonoverlapping peaks of activity correspond to different epitopes.

The total number of distinct epitopes is also likely higher, because only the epitopes eluted in two major first-dimension HPLC peaks of peptides were analyzed. Indeed, preliminary studies in our laboratory indicate that peak A can be resolved in at least five peaks of biological activity from both OVA-6 and SKOV3.A2 cells. A recent study using cultured breast and ovarian tumors reached similar conclusions regarding the potential number of CTL epitopes in breast and ovarian cancer (28). HER-2 peptides found to be active in previous studies of mapping CTL epitopes with synthetic peptides appeared to coelute with the major peaks of CTL activity. Preliminary analysis also indicates that the immunodominant HER-2 peptide (369–377) coeluted with peak B2 in gradient II. Because these CTL lines were previously shown to recognize E75, it is likely that a peptide with similar retention time forming a similar epitope on T2 cells is presented by both the established line SKOV3 and the freshly isolated ovarian tumor OVA-6.

The epitope repertoire identified is only partially overlapping. The fact that none of the effector CTLs was stimulated with either tumor suggests that the effector repertoire was not altered by the tumor epitope recognition. Two categories of epitopes have been identified: (*a*) overlapping (shared), best illustrated by peaks B2, C2, C3, and C4; and (*b*) nonoverlapping, illustrated by peak B1. With respect to the overlapping epitopes, for some, the cytotoxicity values appear to reflect differences in the cell numbers of SKOV3 and OVA-6 tumors of similar levels of MHC class I expression; for others, the distribution is uneven, suggesting that they may be preferentially processed and presented by each tumor. The latter possibility is supported by recent studies indicating that preferential expression of CTL epitopes from the ubiquitous dehydrogenase in different tissues does not correlate with the levels of MHC class I expression (3).

The variable ability of CTL-OVA5 to recognize these epitopes may derive from differential expression of the precursor of these epitopes in the fresh tumors and tumor line (10). An alternative possibility is that the decreased stimulatory ability of the fresh tumor epitopes such as peaks C2, C5, and C6 may reflect altered rates of processing of the same precursor (10, 29). This may reduce epitope generation, leading to decreased recognition by the immune system (29). The presence of nonoverlapping epitopes such as B1 is of particular interest. The fact that SKOV3.B1 is recognized suggests that such an epitope was present and stimulatory on the autologous tumor. The absence from the fresh tumor may suggest immunoselection against those cells expressing SKOV3.B1.

This study, which represents the first comparative analysis of the epitope repertoire presented by a cloned established ovarian tumor line (SKOV3.1E4) and a freshly isolated ascitic ovarian tumor, indicates that a significant number of epitopes presented by the ovarian tumor can be detected in the established tumor line SKOV3. Furthermore a significant number of the peaks of peptides eluted from SKOV3 and OVA-6 are recognized by a human breast CTL line isolated from an HLA-$A2^+$ donor, suggesting that at least some of these epitopes are shared by the breast and ovarian tumors.[5] Thus, with the possible limitations due to the use of allogeneic effectors, these results show that the potential number of CTL epitopes on breast and ovarian tumors is high, and their identification deserves additional investigation. This should be of interest for identification and characterization of tumor Ag in ovarian and breast cancer. Freshly isolated ovarian tumors are difficult to grow, and their establishment in long-term culture, to achieve the desired cell number for this type of study, usually requires specific culture conditions that involve the use of growth factors and stimulation. Thus, the comparative analysis of the epitopes using defined numbers of tumor cells fractionated under defined HPLC conditions should allow identification of bioactive peptides for tumor-associated CTLs and characterization of tumor Ag.

The possibility of characterizing tumor peptides in human ovarian and breast cancer may have important implications for understanding tumor immunity and development of epitope-specific cancer vaccines: (*a*) in contrast to melanoma, in which a large number of tumor Ags have been identified (reviewed by Boon and van der Bruggen; Ref. 30), the number of tumor Ags found in breast and ovarian cancer is significantly smaller. It comprises Muc-1 (31), HER-2 (32), the AES protein of the Notch complex (33) and possibly the folate-binding protein.[6] Identification of additional Ags may allow development of polyvalent tumor vaccines directed to several tumor epitopes. This can minimize the escape of tumor variants and establishment of metastases; (*b*) the use of HPLC-fractionated peptides from established tumor lines may allow us to focus the therapy on the immunogenic epitopes. The active peptide fractions represented less than 10% of the total peptide material eluting from each HPLC column. Their use for vaccination studies will circumvent the blocking of the presenting molecules by inactive peptides. This should increase the Ag density on Ag-presenting cells, a factor that is critical for CTL induction; (*c*) because the presence of CTLs lacking specificity in the OVA-6 tumor infiltrate, as well as the presence of large numbers of tumor cells suggest that this CTL response is ineffective at this tumor stage, vaccine strategies could be developed using these peptides for therapy of earlier stage tumors of smaller size. Epitopes from tumor lines that are recognized by CTL associated with fresh tumors may provide an unlimited source of material for induction of a therapeutic response, bypassing the limitations imposed by the small amounts of fresh tumor cells. In fact, a recent study has demonstrated the ability of acid-eluted peptides to induce curative tumor immunity (14).

ACKNOWLEDGMENTS

We thank Susan Mondragon for outstanding editorial assistance. We also thank Drs. Victor Engelhard and John Lambris for fruitful discussions and advice and Dr. William E. Seifert for mass-spectrometric analysis.

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[CANCER RESEARCH 58, 4790–4793, November 1, 1998]

Advances in Brief

Increased Sensitivity of Adriamycin-selected Tumor Lines to CTL-mediated Lysis Results in Enhanced Drug Sensitivity[1]

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Abstract

The emergence of drug resistance to chemotherapeutic agents is a major cause of treatment failure in cancer therapy. Therefore, much effort has been aimed at circumventing or reversing this undesired effect. Recently, we found that tumor cell lines selected for their multidrug-resistant phenotype can also exhibit increased levels of *TAP* mRNA and MHC class I proteins. This raised the question of whether drug-resistant tumors are more readily recognized by MHC-restricted CTLs. In this report, we show that five of five MHC class I^+ tumor cell lines grown in medium containing Adriamycin developed into variants that expressed higher levels of MHC class I than did their corresponding parental cell lines. This was not observed with a MHC class I^- cell line. No similar association was noted for changes in the expression of either HER-2 or intercellular adhesion molecule 1 protein. We also found that MHC class I^+ drug-selected variants were more readily lysed by MHC-restricted, tumor-associated CTLs than were the drug-sensitive parental cell lines. When the drug-selected variants were cocultured with the same CTLs to eliminate tumor cells expressing higher levels of MHC-I ($MHC\text{-}I^{hi}$), the CTL-resistant tumor cells exhibited a drug sensitivity profile similar to that of the parental cell lines that were not exposed to Adriamycin. These findings suggest that certain chemotherapeutic drugs may increase the immunogenicity of some tumors, and that CTL immunotherapy may help reverse drug resistance.

Introduction

Prolonged exposure of tumor cells to cytotoxic chemotherapeutic drugs such as ADR,[3] etoposides, and *Vinca* alkaloids leads to the development of the MDR phenotype, which results in resistance to various types of drugs (1). The development of multidrug resistance plays a major role in the failure of treatment of many types of cancers. Consequently, much effort has been directed at both understanding its development and deriving the means to reverse or circumvent its effects. Still, this problem represents a major obstacle to progress in cancer therapy.

Two different proteins are known to mediate multidrug resistance activity: (*a*) P-glycoprotein, the product of the *MDR1* gene (2); and (*b*) MRP (3). These proteins are thought to act as energy (ATP)-dependent efflux pumps that prevent the intracellular accumulation of cytotoxic compounds. Both proteins belong to the ABC superfamily of transmembrane transporters, the family that also includes the TAP proteins (4–6). TAP is a heterodimer that transports peptides from the cytosol into the endoplasmic reticulum, where they are available for binding to the MHC class I heavy chain (7). Such presentation of antigenic peptides is a prerequisite for the recognition and lysis of infected or transformed cells by CTLs.

Because of the structural and functional similarities between the genes associated with MDR and TAP, we recently investigated whether MDR tumor cells also have altered peptide transport systems (8). We found that the development of the MDR phenotype was paralleled by an increased accumulation of *TAP* mRNA, resulting in a higher level of MHC class I expression relative to that of the parental cell lines. These findings were recently confirmed by Izquierdo *et al.* (9), who also found both TAP and MHC class I to be overexpressed in several MDR tumors.

The findings of enhanced antigen-presenting capabilities among MDR tumors raised questions about the immune recognition of drug-resistant cells in comparison to their drug-sensitive counterparts. It has been demonstrated in experimental models that anticancer drugs, although often thought of as immunosuppressive, can actually potentiate a variety of immune responses (*e.g.,* delayed-type hypersensitivity and abrogation of tolerance; Ref. 10). One of the most widely studied chemotherapeutic agents in this regard is cyclophosphamide (reviewed in Ref. 10). The immunopotentiation observed with cyclophosphamide is thought to result from the inhibition/depletion of suppressor T cells and may be observed with the administration of cyclophosphamide before tumor challenge (10, 11). It has also been shown that the administration of chemotherapeutic agents such as melphalan can result in increased tumor infiltration by $CD8^+$ T lymphocytes with potent, antigen-specific cytotoxic activity *in vitro* (12). ADR was found to result in a dose-dependent increase in tumor-specific CTL activity in mice receiving tumor cell vaccines, particularly when it was administered 1 week after vaccination as opposed to administration before vaccination (13). Furthermore, the development of regimens that alternate cytotoxic therapy with immunotherapy (sequential chemoimmunotherapy) has demonstrated a synergystic effect of the two modalities in clinical trials (14, 15). The exact mechanism by which chemotherapy induces this immunopotentiation remains to be elucidated. We hypothesized that: (*a*) the increased expression of TAP and MHC class I proteins associated with the MDR phenotype renders such tumor cells more susceptible to recognition and lysis by MHC class I-restricted, tumor-specific CTLs; and (*b*) the elimination of the MHC^{hi} cells within a population of MDR cells results in increased sensitivity of the remaining population of cells to the cytotoxic effects of chemotherapeutic drugs by also eliminating the MDR- or MRP-overexpressing cells.

Materials and Methods

Fluorescence-activated Cell-sorting Analysis. Tumor surface antigens were detected as described previously (16), using an EPICS V Profile Analyzer (Coulter Corp., Hialeah, FL). Antibodies to HLA ABC (W6/32; DAKO, Glostrup, Denmark), HER-2/neu (Ab2; Oncogene Science, Manhasset, NY),

Received 7/22/98; accepted 9/18/98.

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[1] Supported by a scholarship from the Health Professions Program provided by the United States Department of the Army (to B. F.) and by Grant DAMD 17-94-J-4313 (to C. G. I.).

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[3] The abbreviations used are: ADR, Adriamycin; MDR, multidrug-resistant; TAP, transporter associated with antigen processing; ICAM, intercellular adhesion molecule; ABC, ATP-binding cassette; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IL, interleukin; HLA, human lymphocyte antigen; NFκB, nuclear factor κB.

and ICAM-1 (Calbiochem, San Diego, CA) were not conjugated. Cells to be examined were incubated with the appropriate antibody at 4°C for 30 min, washed, and further incubated with goat antimouse IgG (Boehringer Mannheim, Indianapolis, IN). Cells were washed again after 30 min and then analyzed.

CTL Cytotoxicity Assays. Cytotoxic activity of tumor-associated lymphocytes/CTLs was determined using the *in vitro* ^{51}Cr release assay (16). CTLs used as effectors were generated as described previously (16, 17). For the cytotoxicity assay, 1–2 $\times$ 10^6 target cells were labeled with 100 μCi of ^{51}Cr (Amersham, Arlington Heights, IL) at 37°C for 90 min, washed three times, and plated in triplicate at a final concentration of 5 $\times$ 10^3 cells/well in 96-well V-bottomed microtiter plates (Costar, Cambridge, MA) containing the appropriate number of effector cells. For MHC class I inhibition, 5 μl of W6/32 were added to the appropriate wells. Maximum release was obtained by adding 0.1N HCL. The percentage of specific target cell lysis was determined by the following formula:

$$\frac{\text{Experimental } ^{51}\text{Cr release} - \text{Spontaneous release}}{\text{Maximum } ^{51}\text{Cr release} - \text{Spontaneous release}} \times 100$$

Drug Selection. Drug-selected variants were derived from breast (SKBR3, MCF-7, and MDA MB453) and ovarian (SKOV3, MDA 2774, and CaOV3) tumor cell lines by exposure to gradually increasing concentrations of ADR. In brief, 1 $\times$ 10^6 cells were seeded in T-25 flasks with 12 ml of RPMI-FCS [RPMI 1640 (Life Technologies, Inc.) + 10% FCS + 40 μg/ml gentamicin]. ADR (Sigma) was added at a final concentration of 1 ng/ml. Cultures were split every 3–4 days, at which time the ADR concentration was increased. Concentrations were increased from 1 ng/ml to 2, 4, 10, 15, 20, . . . 100 ng/ml over a 1-month period. ADR-selected tumor cells were 100% viable in 125 ng/ml ADR by the MTT assay. Nonselected parental cell lines were cultured and split simultaneously.

Immunoselection. CTL escape variants were generated from drug-selected tumor cell lines as follows. Cells of the line to be selected were added at 5 $\times$ 10^4 cells/well to a 24-well Falcon plate along with 2.5 $\times$ 10^5 CTLs (CTL-B or CTL-E) in a final volume of 2 ml of RPMI-FCS + 50 units/ml IL-2 (Cetus). A similar number of seeded wells were incubated without IL-2 (RPMI-FCS alone) as controls. After 2 days, the wells were rinsed gently with RPMI 1640, and the nonadherent cells were removed. Both immunoselected and control wells were then further cultured in RPMI-FCS without IL-2 and split as needed. The determination of drug sensitivity was performed 7 days after the initiation of selection with CTLs.

Drug Sensitivity Assay. The assay was modified from that described by Wilson *et al.* (18). Cells were added at 2,5 $\times$ 10^4 cells/well in a final volume of 100 μl of RPMI-FCS to a 96-well flat-bottomed plate, which already contained triplicate dilutions of ADR. Final dilutions of ADR ranged from 4 to 500 ng/ml. Plates were incubated at 37°C for 18 h, after which 25 μl of MTT (at 2.5 mg/ml) was added per well. The plates were incubated for an additional 4 h and then centrifuged for 5 min at 200 $\times$ *g*. Medium and unconverted MTT were removed by inversion, and 75 μl of DMSO were added to each well. Plates were incubated on a rotator for 10 min and then read at 570 nm by a Dynatech auto plate reader. The effect of the cytotoxic drug was determined by calculating the absorbance of the test wells as a percentage of that of the control wells.

Results

ADR-selected Tumor Cell Lines Exhibit Increased Levels of MHC Class I. To examine the effect of chemotherapeutic resistance on tumor MHC class I expression, we exposed six established tumor cell lines (three ovarian and three breast cancer cell lines) to increasing concentrations of ADR. The levels of MHC class I expression of the ADR-selected variants were then determined by fluorescence-activated cell-sorting analysis and compared with those of the corresponding drug-sensitive parental cell lines. Increased levels were found in all five ADR-selected variants that had corresponding MHC class I^+ parental cell lines (Table 1). The levels of increased MHC class I expression varied from a 14% increase observed with SKOV3 to a 156% increase seen with MCF-7. The remaining cell lines exhibited between a 40 and 80% increase in MHC class I expression. One cell line, MDA 2774, consisted of two distinct populations expressing high and low levels of MHC class I. Both populations in the ADR-selected variant, 2774-DR, exhibited increased levels of MHC class I.

Table 1 *Increases in HLA-A, -B, and -C antigen expression in drug-resistant tumor cell lines*

Tumor cell line	Mean level of fluorescence: Drug sensitive	Drug resistant	MCF-R[a]
HLA class I			
MDA 2774	155	215	1.39
SKOV3	190	216	1.14
CaOV3	194	346	1.78
MCF-7	80	205	2.56
SKBR3	80	120	1.50
MDA MB453	44[b]	44[b]	1.0
HER-2/neu			
MDA 2774	153	153	1.0
SKOV3	385	396	1.0
CaOV3	10	10	1.0
MCF-7	335	320	0.95
SKBR3	139	139	1.0
MDA MB453	613	536	0.87
ICAM-1			
MDA 2774	230	190	0.82
SKOV3	111[b]	119[b]	1.27
CaOV3	78	99	1.27
MCF-7	275	274	1.0
SKBR3	50	60	1.0
MDA MB453	116	119[a]	1.0

[a] MCF-R, mean channel fluorescence ratio was obtained by dividing each value in the second column by the corresponding value in the first column (*i.e.*, 215:122 = 1.39).
[b] Negative samples (antibody-stained population showed no difference in comparison to the negative control).

One parental tumor cell line, MDA MB453, which was negative for MHC class I expression, was used as a control. The corresponding ADR-selected variant, MB453-DR, was the only drug-selected cell line that did not show any changes in MHC class I expression. Hence, the loss of MHC class I expression is not corrected by selection with ADR or the development of drug resistance.

Increased expression of the proto-oncogene HER-2 has also been described as being associated with MDR1-overexpressing breast and ovarian tumors (19). Because increased HER-2 expression results in CTL recognition (17), we also determined the levels of HER-2 expression on the ADR-selected variants. As shown in Table 1, HER-2 expression was slightly increased in one cell line, unchanged in three cell lines, and decreased in two other cell lines. We also examined for differences in ICAM-1 expression between drug-selected and nonselected tumor cells, because this adhesion molecule can facilitate tumor recognition by cellular immune effectors. Two of the six tumor cell lines were negative for ICAM-1 expression, as were the corresponding drug-selected variants. Of the four ICAM-1-positive cell lines, two showed an increase in ICAM-1 expression in the drug-selected variants, one showed no change, and one showed a slight decrease. Thus, whereas MHC class I expression was clearly increased in all ADR-selected variants, HER-2 and ICAM-1 expression showed no such association.

Increased CTL-mediated Lysis of ADR-selected Tumor Cell Lines. CTL cytotoxicity assays were performed to determine whether the increased levels of MHC class I expression in ADR-selected variants resulted in increased target sensitivity to lysis. To ensure that the results were relevant for several HLA types, we used three ovarian CTL lines that express at least one HLA in common with the MDA 2774 cell line (CTL-B, HLA-A3; CTL-E and CTL-R, HLA-A24) as effectors. These CTL cell lines have been previously shown to preferentially lyse autologous tumors (15). All three CTL cell lines lysed

the parental MDA 2774 cells, but a higher lysis of the ADR-selected 2774-DR variant than of the parental cell line was observed (Fig. 1, *A* and *B*). Lysis was inhibited by the addition of the anti-MHC class I antibody W6/32 (Fig. 1*B*). As expected, neither the MHC class I^- MDA MB453 nor the MB453-DR tumors were lysed, indicating that the increased sensitivity to lysis is dependent on MHC class I expression, and that tumor lysis by these effectors is not likely to be the result of a natural killer-lymphokine-activated killer cell activity.

A fourth CTL cell line, CTL-V (HLA-11, B60, 62), was tested against SKBR3 (HLA-A11, B18, 40) and its ADR-selected variant, SKBR3-DR. Neither SKBR3 nor SKBR3-DR was lysed by CTL-V (data not shown). Because both targets shared HLA-A11 but expressed lower levels of MHC class I than did the other tumor cell lines tested (Table 1), we retested CTL-V-mediated lysis after pretreating the tumors with 300 units/ml IFN-γ for 24 h. However, the IFN-γ-treated tumors were still resistant to lysis (data not shown). These results suggest that CTLs that lack antigen recognition of the ADR-sensitive tumor will not recognize the ADR-selected tumor. Drug selection did not appear to alter the antigen profile of the tumor as recognized by these effectors but merely increased the antigen presentation.

Immunoselection with CTLs of ADR-selected Variants Increases Drug Sensitivity. On the basis of findings of increased sensitivity of ADR-selected variants to CTL-mediated lysis, we hypothesized that selection by the CTLs may result in the elimination of those tumor cells with greater drug resistance potential. If this hypothesis is correct, then the resulting population of CTL escape tumor variants would then be more susceptible to the cytotoxic activity of ADR. To test this hypothesis, we derived CTL escape variants by coculturing the drug-selected cell lines with CTLs. We then compared the ADR sensitivity of the CTL escape variants with that of both the non-CTL-selected drug-resistant variants (cultured for the same interval in the absence of ADR) and the drug-sensitive parental cell lines in MTT assays. As shown in Fig. 2*A*, the non-CTL-selected 2774-DR cell line was resistant to the cytotoxic activity of ADR up to concentrations of 125 ng/ml. The parental MDA 2774 cell line exhibited sensitivity at ADR concentrations as low as 8 ng/ml. Interestingly, the CTL-resistant variant derived from 2774-DR by selection with CTL-B exhibited an ADR sensitivity profile that was indistinguishable from that of the parental MDA 2774 cell line.

We repeated the experiment with CTL-E and the SKOV3-DR cell line, which share HLA-B35. Based on the MTT assay, the parental SKOV3 cell line appears to be more inherently resistant to ADR than MDA 2774, exhibiting sensitivity only at high concentrations of ADR ($\geq$250 ng/ml; results not shown), similar to the profile of the drug-selected SKOV3-DR cell line. Thus, the pattern of sensitivity to ADR was in the range of 4–125 ng/ml (Fig. 2*B*). This may be a reflection of prior *in vivo* selection with chemotherapeutic drugs, which is supported by the observation that among the MHC class I^+ cell lines, SKVO3 exhibited the lowest increase in MHC class I expression with ADR exposure (Table 1). Of interest, the CTL-resistant variant of SKOV3-DR was sensitive to much lower concentrations of ADR than were the SKOV3 and SKOV3-DR cell lines. Thus, our findings suggest that CTL-mediated lysis could eliminate those cells within a tumor population that are more resistant to ADR, leaving a more susceptible population.

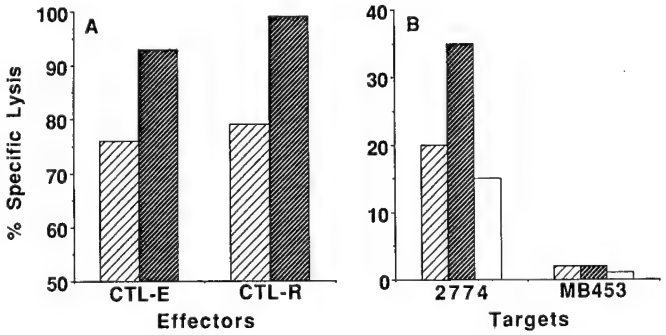

Fig. 1. Increased susceptibility of the drug-selected MDA 2774 tumor cell line to CTL-mediated lysis. CTL-E and CTL-R (*A*) and CTL-B (*B*) were tested for lysis of unselected (▨) and ADR-selected (▨) 2774 tumor cell lines. Additionally, CTL-B was tested against the ADR-selected MDA 2774 cell line in the presence of anti-MHC class I antibody (□) as well as against the MHC class I^- cell line MDA MB453 (*B*).

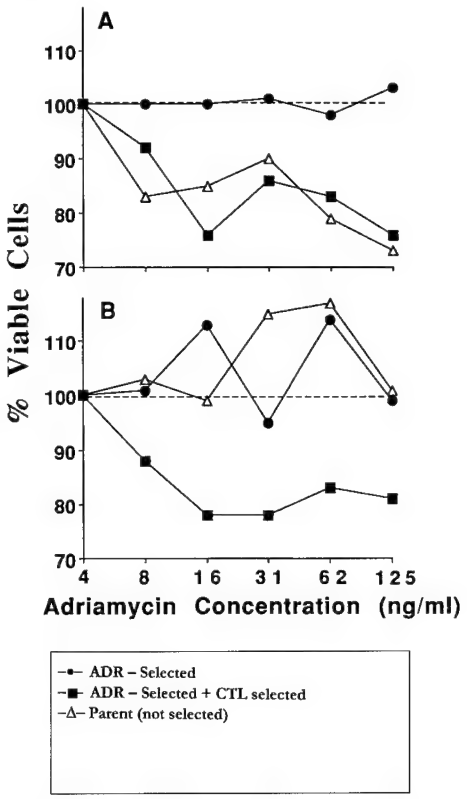

Fig. 2. Increased drug-sensitivity in CTL-resistant variants of the ADR-selected lines. ADR-selected MDA 2774 (*A*) and SKOV3 (*B*) cells were cultured in the presence (■) or absence (●) of HLA-matched CTLs (CTL-B and CTL-E, respectively) for 7 days. Parental cell lines without previous ADR exposure (△) were cultured under identical conditions as the nonimmunoselected drug-selected cell lines. The subsequently derived tumor cell lines were examined in drug sensitivity assays as described in "Materials and Methods."

Discussion

In this report, we present novel evidence that the development of resistance to chemotherapeutic agents such as ADR is associated with the increased susceptibility of tumors to CTL lysis. This is paralleled by an increase in MHC class I expression. Increased levels of MHC class I associated with drug selection resulted in an increased sensitivity to CTL-mediated lysis. Lysis was MHC restricted and required MHC expression. Lysis also required the tumor to present peptide antigen to be recognized by TCR. This was suggested by the finding that CTL-V, which could not lyse the MHC class I^+ SKBR3 parental cell line, was also unable to lyse the drug-resistant SKBR3-DR cell line, even after pretreatment with IFN-γ. Therefore, the increased sensitivity to lysis of drug-resistant variants seems to require both an intact antigen presentation pathway and the presence of antigen recognized by effectors.

Immunoselection by coculture of drug-selected tumors with CTLs resulted in the reversion of the surviving tumor cells to a more

drug-sensitive status. However, this did not reflect a spontaneous reversion of escaping tumors to a sensitive phenotype, because the same cells cultured in the same conditions without CTLs were far more resistant to ADR. Thus, the induction of reversion of drug sensitivity is likely the result of the elimination of those tumors expressing the MDR phenotype with a concomitantly higher level of MHC class I. If exposure to a chemotherapeutic drug selects for tumor cells with increased expression of both the MDR phenotype and MHC class I, it follows that after the elimination of those cells, the remaining population of cells will have a lower potential for expressing the MDR phenotype. The mechanisms involved in the increased TAP/MHC class I expression are not known but may involve common transcription factors and intermediates (adapter proteins) also used by the proteins encoded by the drug resistance genes MDR and MRP. NFκB has recently been demonstrated to be involved in the transcriptional regulation of genes in the *mdr* family (19) as well as the regulation of TAP1 expression (20). NFκB activity can be induced by a variety of stimuli, including cytotoxic compounds and other cellular stressors (21). The concomitant induction of stress response NFκB transcription factors in response to cytotoxic stress may therefore play a role in the increased expression of both *mdr* and TAP. Moreover, the Raf-1 kinase, which activates NFκB, was also found to be involved in *mdr* expression (21, 22).

It is important to emphasize that MHC class I expression does not appear to be required for the development of the drug-resistant phenotype induced by ADR. The absence of a requirement for MHC class I is demonstrated by the ability to select the MHC class I^- cell line MDA MB453 in ADR. The drug-selected variant was MHC class I^- as well. Thus, genetic changes resulting in the development of drug resistance do not seem to result in changes that can compensate for existing genetic defects in MHC class I expression. We also did not find increased expression of HER-2 and ICAM-1, making it unlikely that these play a role in the increased lysis of drug-selected tumors by CTLs.

Together, our findings suggest a possible mechanism for synergy between chemotherapeutic agents and immunotherapy. Chemotherapy may decrease the tumor burden, but the remaining tumor cells, as shown here, will express increased levels of MHC class I. These remaining cells are then more likely to be recognized and eliminated by tumor-specific CTLs. Furthermore, CTLs may then eliminate those tumor cells with a higher potential for chemotherapeutic resistance, thus sensitizing the tumor population for a subsequent round of chemotherapeutic drug exposure. Repeated rounds of sequential chemoimmunotherapy may thus result in enhanced responses through greater reductions in tumor burdens.

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Vaccine Implications of Folate Binding Protein, a Novel Cytotoxic T Lymphocyte-recognized Antigen System in Epithelial Cancers[1]

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ABSTRACT

The immune system can be efficiently stimulated and targeted to specific antigens expressed exclusively or preferentially by experimental cancers. The foremost limitations to extending this vaccine technology to the prevalent epithelial-derived cancers are the lack of: (*a*) identified tumor-associated antigens recognized by cellular immunity; (*b*) antigens expressed on the majority of tumor cells during disease progression; and (*c*) immunogenic CTL epitopes. To date, only HER-2/neu has been shown to be the source of naturally occurring, MHC-restricted, CTL-recognized peptides in epithelial tumors. In this study, we demonstrate that the human high-affinity folate binding protein (FBP), which is a source of antigenic peptides recognized in ovarian cancer, is also recognized in breast cancer. Both immunodominant E39 (FBP, 191–199) and subdominant E41 (FBP, 245–253) epitopes are presented by HLA-A2 in these cancers. These peptides are efficient at amplifying the response of tumor-associated lymphocyte populations in terms of lytic function, enhanced proliferation, and specific IFN-γ release. On a per cell basis, tumor-associated lymphocytes stimulated with the FBP peptides exhibit enhanced cytotoxicity not only against peptide-loaded targets but also against FBP-expressing epithelial tumors of different histologies. Furthermore, FBP peptides induced E39-specific CTLs and E39- and E41-specific IFN-γ and IP-10 secretion in certain healthy donors. The broad distribution of FBP among >90% of ovarian and endometrial carcinomas, as well as 20–50% of breast, lung, colorectal, and renal cell carcinomas, along with pronounced differential overexpression in malignant tissues compared with the extremely limited expression in normal epithelium, suggests the exciting potential of a widely applicable FBP-based vaccine in epithelial cancers.

INTRODUCTION

Anticancer vaccines have taken many forms, from whole tumor cells and viral oncolysates, to most recently, Ag[3]-specific, peptide-based vaccines (1–3). The practical advantage of the former is that the actual CTL-recognized Ags need not be known. The latter approach requires precise knowledge of the tumor Ag recognized by specific antitumor CTLs (3). For vaccination purposes, TAAs may be partially purified, highly purified, or synthetic in nature. The synthetic peptide Ag TAA vaccines (or genes encoding for these Ags) should theoretically offer the best cancer vaccines by delivering the immunogenic Ag(s) capable of inducing an efficient, specific, tumor-protective immunity without interference from irrelevant Ags. The TAA vaccines can be easily and reproducibly manufactured and delivered in high volumes safely.

To extend the advances in vaccine technology to the vast problem of epithelial cancers, CTL-recognized TAAs must be identified first. Such TAAs should also meet the criteria of high and stable expression in tumor cells of a large number of patients during disease progression to allow targeting. To date, only the protein product of the proto-oncogene *HER-2* and the core peptide of MUC-1 have been shown to be a source of CTL-recognized peptides (4–7). The advantage of the HER-2 Ag system over those known for melanoma is that it is expressed in multiple epithelial-derived tumor histologies like breast, ovarian, pancreas, and non-small cell lung carcinomas, making a HER-2 peptide-based vaccine potentially widely applicable (8–12). However, this protein is only overexpressed in 30% of breast and ovarian cancers and less in others (13).

FBP is a membrane-associated glycoprotein originally found as a mAb-defined Ag in placenta and trophoblastic cells but rarely in other normal tissues (14–17). Of interest, this protein has been found in >90% of ovarian and endometrial carcinomas; in 20–50% of breast, colorectal, lung, and renal cell carcinomas; and in multiple other tumor types. When present in cancerous tissue, the level of expression is usually >20-fold normal tissue expression and has been reported to be as high as

Received 5/18/99; revised 8/20/99; accepted 9/5/99.
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[1] This work was supported in part by Grant DAMD-17-94-J-4313 (to C. G. I.). Peptide synthesis was supported in part by Core Grant CA 16672.
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[3] The abbreviations used are: Ag, antigen; TAA, tumor-associated Ag; FBP, folate binding protein; mAb, monoclonal antibody; TAL, tumor-associated lymphocyte; OvTAL, ovarian cancer-associated TAL; BrTAL, breast cancer-associated TAL; HER-2, HER-2/neu; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; IL, interleukin; APC, Ag-presenting cell.

80–90-fold in ovarian carcinomas (18). Although FBP has been investigated extensively as a target of humoral immunity, it has only recently been proposed as a source of CTL-recognized peptides in ovarian cancer (19). To investigate the potential role of FBP as a TAA vaccine, we studied whether these peptides are antigenic for BrTALs and immunogenic in ovarian cancer patients and healthy donors. We investigated whether FBP peptides can amplify CTL-TALs with broad tumor-killing capabilities and activate CTLs from healthy donors.

In this study, we demonstrate that two FBP peptides, E39 (191–199) and F41 (245–253), are recognized by freshly cultured OvTALs and by BrTALs. These peptides correspond to naturally processed Ags on intact tumor cells. Additionally, FBP peptides E39 and E41 are capable of CTL stimulation *in vitro,* resulting in proliferation, peptide-specific cytokine and chemokine release, and enhanced cytotoxicity. E39-stimulated specific CTL-TALs are capable of lysing multiple tumors with different epithelial-derived histologies. Furthermore, E39 could stimulate PBMCs to induce E39-specific CTL activity when presented on DCs from healthy donors. Demonstration of E39 and E41 immunogenicity may be significant for development of TAA vaccines based on FBP.

MATERIALS AND METHODS

TAL Cultures. TALs were isolated from fresh collections of malignant ascites and pleural effusions from four ovarian and two breast cancer patients, respectively, under the approval of the Institutional Review Board. Specimens were processed as described previously (20). Lymphocytes and tumor cells were separated by centrifugation over discontinuous 75%/100% Ficoll-Histopaque (Sigma Chemical Co., St. Louis, MO) gradients. Freshly isolated TALs were suspended in RPMI 1640 containing 100 μg/ml L-glutamine (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Sigma), 40 μg/ml gentamicin, and 50–100 IU/ml IL-2 (Cetus, Emeryville, CA), cultured at 0.5–1.0 × 10^6 cells/ml in a humidified incubator at 37°C in 5% CO_2, and maintained at this concentration by the addition of media and IL-2 every 2–3 days, depending on the growth kinetics.

Tumor Targets. The SKOV3 ovarian carcinoma cell line was transfected with the HLA-A2 expression vector RSV.5-neo with resulting high levels of HLA-A2 expression (SKOV3.A2) as described (21). The SKBr3 breast cancer line was similarly transfected with the *HLA-A2* gene (by Drs. M. Disis and M. Cheever, University of Washington, Seattle, WA) but expresses moderate levels of HLA-A2 (SKBr3.A2) when compared with SKOV3.A2. Both lines were maintained in RPMI 1640 with 10% FCS and 250 μg/ml G418 (Sigma). SW480 is an established, well-characterized HLA-$A2^+$ colon cancer cell line, and PAN-1 is a HLA-$A2^+$ pancreatic cancer cell line. SKBr3.A2, SKOV3.A2, and SW480 express HER-2 with decreasing levels in this order. SKOV3.A2, SW480, and Panc-1 have been shown to express FBP (17, 19).

Phenotype Analysis. The HLA-A2 status of the TALs and tumor lines was determined by indirect staining with anti-HLA-A2 mAbs, BB 7.2 and MA 2.1 (American Type Culture Collection), followed by goat anti-mouse mAb conjugated with FITC (Becton Dickinson, Mountain View, CA), and analyzed on a Coulter Epics C Flow Cytometer (Coulter Electronics, Hileah, FL). HER-2 expression was tested similarly using the Ab2 mAb (Oncogene Science, Manhasset, NY). FBP expression was analyzed using the MOv18 mAb generously donated by Centocor (Malvern, PA).

Synthetic Peptides. The FBP sequence was interrogated for potential HLA-A2-binding nonamers using the known binding motifs for this molecule (22). Five peptides were selected for synthesis based on the presence of leucine, isoleucine, or valine in the dominant anchor positions, P2 and P9, and their potential to form amphiphilic helices (19). Peptides were prepared by the Synthetic Antigen Laboratory of University of Texas M. D. Anderson Cancer Center. Identity and purity of final materials were established by amino acid analysis and analytical reverse phase-high performance liquid chromatography. All peptides used in this study were between 92 and 95% pure. The symbols, position, and sequence of the peptides used in this study are as follows: E37 (FBP, 25–33) RIAWARTEL; E38 (FBP, 112–120) NLGPWIQQV; E39 (FBP, 191–199) EIWTHSTKV; E40 (FBP, 247–255) SLALMLLWL; and E41 (FBP, 245–253) LLSLALMLL. All of these peptides are low to moderate binders, except E38, which is a high-affinity binder to HLA-A2. The low-affinity HLA-A2 binding peptide E71 (HER-2, 798–807) QLMPYGCLL and the CTL epitope E75 (HER-2, 369–377) were used as specificity controls (5).

Cytotoxicity Assays. Cytotoxicity was determined by standard chromium-release assays as described previously (20). Briefly, tumor targets were labeled with 100–150 μCi of sodium chromate (^{51}Cr; Amersham, Arlington Heights, IL) for 1.5 h at 37°C, washed twice, and plated at 2000–2500 cells/well in 100 μl in 96-well, V-bottomed plates (Costar, Cambridge, MA). Effectors were added at designated E:T ratios in 100 μl/well. After 5–20 h of incubation, 100 μl of culture supernatant were collected, and ^{51}Cr release was measured on a γ-counter (Gamma 5500B; Beckman, Fullerton, CA). All determinations were done in triplicate or tetraplicate. Results are expressed as percentage of specific lysis as determined by:

$$\% \text{ specific lysis} = \frac{\text{Experimental mean cpm} - \text{spontaneous mean cpm}}{\text{Maximum mean cpm} - \text{spontaneous mean cpm}} \times 100$$

For peptide-pulsed cytotoxicity assays, the T2 line (generously donated by P. Creswell, Yale University, New Haven, CT) was used as target. T2 is a human T-cell/B-cell fusion product containing an Ag-processing defect in the transporter-associated proteins such that HLA-A2 molecules are empty on the cell surface or contain relatively few bound peptides that can be effectively displaced by exogenous HLA-A2-binding peptides (23). The T2 cells were labeled with ^{51}Cr as above, washed, and then incubated with peptide for 1.5 h at 37°C prior to standard cytotoxicity assays. To increase the sensitivity of detection at low E:T ratios by uncloned effectors, 20-h CTL assays were used in parallel with the 5-h assays. T2 without peptide (T2-NP) was also used as a control. For cold target inhibition assays, unlabeled T2 were incubated with peptide for 1.5 h and then added to standard cytotoxicity assays with chromium-labeled tumor targets and effectors. The cold:hot target ratio was 15:1.

CTL Induction Experiments. Freshly cultured TALs were plated at 1×10^6 cells/ml in 24-well culture plates (Costar) in RPMI 1640/10% FCS without IL-2. T2 cells were irradiated with 100 Gy (Cesium source), washed, and incubated with saturating concentrations of individual FBP peptides for 1.5 h prior to being added to six parallel TAL cultures at a 10–15:1 responder:stimulator ratio. After 48 h, 50 IU/ml of IL-2 were added. Medium and IL-2 were then added every 2–3 days as needed. Parallel control cultures were established with T2-NP in the exact same manner. After 1 week in culture, cells were counted, and the proliferation index was calculated as a ratio of peptide-stimulated culture cell number to the control cultures stimulated with T2-NP. This approach was preferred over DNA synthesis (thymidine incorporation) because it also indicates the viability of the cells after stimulation.

For stimulation with E39 presented on DCs, PBMCs were collected from three healthy HLA-$A2^+$ donors. DCs were generated by the CD14 method, *i.e.,* from plastic adherent PBMCs after culture in 1000 units/ml granulocyte/macrophage-colony stimulating factor and 500 units/ml of IL-4. DCs generated by this method express the CD13 marker, high levels of MHC-I, MHC-II, CD86, and CD-54, moderate levels of CD40, and low levels of CD80 (24, 25). This phenotype is consistent with immature DCs, which can uptake large amounts of Ags. DCs were pulsed with E39 at 50 μg/ml in serum-free medium, followed by tumor necrosis factor α (50 units/ml). DC-E39 were used as stimulators for autologous plastic nonadherent PBMCs at a ratio of 1:25 (stimulator:responder). IL-2 was added 24 h later in all cultures at 60 IU/ml. Cytotoxicity assays were performed as above at 1 week from the last stimulation.

Cytokine Assays. Peptide-stimulated, parallel-cultured TALs were replated at 1×10^6 cells/ml after 1 week and restimulated in the same fashion as described above. Supernatants from the parallel cultures were harvested at 24 and 48 h prior to the addition of IL-2 and stored at −20°C until analyzed. Supernatants from DC-E39-stimulated PBMCs were also collected at 24 and 48 h. Peptide-specific cytokine release from the TALs was measured in 50 μl for IFN-γ and IL-4 at the two time points using ELISA kits (BioSource, Camarillo, CA) with a sensitivity of 4 pg/ml according to the manufacturer's instructions. Results are given as pg/ml produced by 1×10^6 cells.

IP-10 ELISA. The ability of cells to secrete IP-10 in response to FBP peptides was determined by culturing PBMCs and collecting supernatants at corresponding times. The levels of IP-10 secreted were determined using a modified sandwich ELISA (R & D Systems, Minneapolis, MN). A flat-bottomed, 96-well microtiter plate was coated with 100 μl/well of monoclonal anti-human IP-10 (2 μg/ml in PBS, pH 7.2) for 24 h at room temperature. The plate was subsequently washed with PBS (pH 7.4), 0.05% Tween 20 and then blocked with 3% ovalbumin, 5% sucrose, and 0.05 NaN_3. IP-10 standards were made from recombinant human IP-10 in a solution consisting of Tris-buffered saline (TBS; pH 7.3), 0.05% Tween 20, and 0.1% BSA using serial dilutions. One hundred μl/well of the standards and the cell supernatants were plated in duplicate and left at room temperature for 2 h. After washing the plate three times, 100 μl/well of biotinylated monoclonal anti-human IP-10 [100 ng/ml in TBS (pH 7.3), 0.1% BSA] was added, followed after washing by 100 μl/well of streptavidin-peroxidase conjugate.

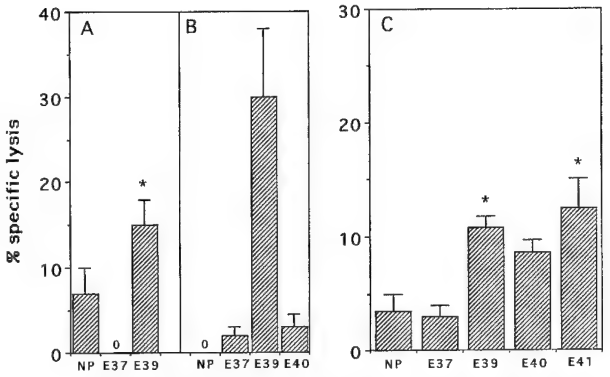

Fig. 1 Freshly cultured OvTALs and BrTALs recognize FBP peptides. *A,* HLA-$A2^+$ BrTAL-1 isolated from a pleural effusion, cultured in IL-2 without specific stimulation, was tested for recognition of FBP peptides E37, E39, and E40 (*B*). The experiments shown in Fig. 1*A* were performed using the same culture as effectors at 8 days from each other. Peptides were used at 25 μg/ml in *A* and 50 μg/ml in *B*. E:T ratios were 20:1 and 30:1, respectively. *C,* OvTALs were tested for recognition of FBP peptides (E37-E41) or no peptide (T2-NP) as a negative control. The 5-h ^{51}Cr-release assays were performed in triplicate at an E:T ratio of 20:1 and repeated two to four times for each effector. Pooled data with four OvTAL populations in 12 independent assays demonstrate E39 to be the most consistently recognized FBP peptide. E40 and E41 were recognized by some TALs ($n = 2$) but not others. The results are expressed as percentage of specific lysis (*, $P < 0.05$ *versus* NP); *bars,* SE.

Chromogen substrate, 100 μl/well, consisted of DMSO and H_2SO_4. Plates were read at 450 nm in an automated microplate reader (Bio-Tek Instruments, Inc., Richmond, CA). Standard dilutions of IP-10 ranged from 4000 to 15.6 pg/ml. This method consistently detected IP-10 concentrations >31.25 pg/ml in a linear fashion.

RESULTS

Ovarian and Breast Cancer-associated Lymphocytes Recognize FBP Peptides. The isolated TALs from breast cancer patients were cultured in medium containing IL-2 without restimulation with autologous tumor. Cytotoxicity assays using peptide-loaded T2 cells as targets were performed with the TALs within 7–14 days of culture initiation to limit the *in vitro* expansion of irrelevant clones. One of two BrTALs, designated BrTAL-1, recognized E39. The results in Fig. 1*A* show specific recognition by BrTAL-1 of FBP peptide E39 compared with another FBP peptide, E37, or control T2 cells, which were not pulsed with peptide (T2-NP). The presence of CTLs with this epitope specificity was confirmed by retesting the E39 recognition 8 days later (Fig. 1*B*) at higher E:T ratio (30:1) and higher Ag concentration (50 μg/ml). These results indicated that the E39 specificity is present *in vivo* among the breast-associated CTLs and stable within the first 3 weeks of culture.

OvTALs were also screened for FBP peptide reactivity. Four of four OvTALs recognized FBP peptides. We found that among the FBP peptides, E39 was recognized most consistently by the four OvTALs. Fig. 1*C* shows the pooled results of experiments using as effectors fresh OvTAL cultures. E37, a low affinity binder, and E38, a high affinity binder, were not

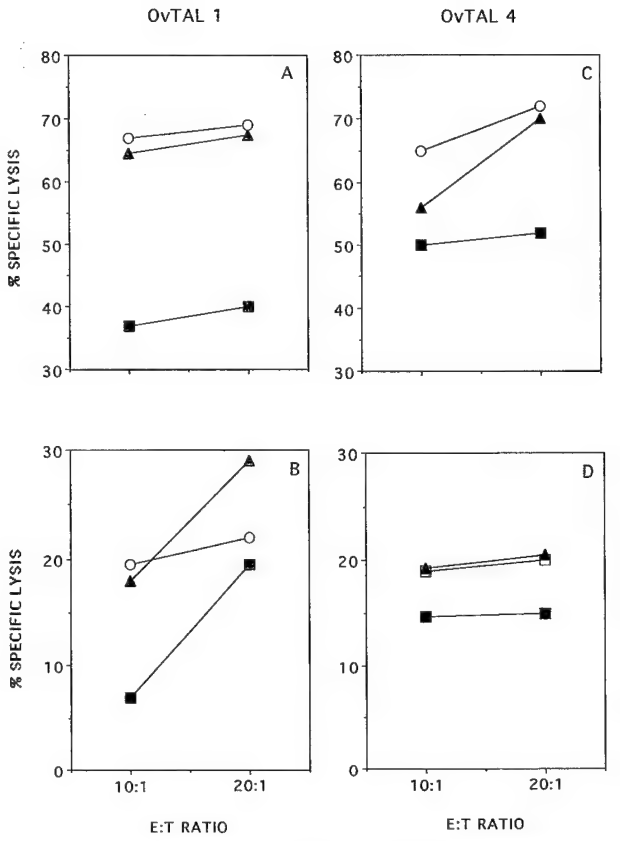

Fig. 2 E39 (FBP, 191–199) reconstitutes an epitope corresponding to a naturally processed and presented Ag in ovarian cancer. Cold target inhibition assays were performed with OvTAL-1 (*A* and *B*) and OvTAL-4 (*C* and *D*). T2 loaded with E37 (negative control peptide; ▲), E39 (■), or no peptide NP (○, □) were tested at a cold:hot ratio of 15:1 for inhibition of the recognition of the ovarian cancer cell line SKOv3.A2 by OvTALs at E:T ratios of 10 and 20:1 in 5-h (*B* and *D*) and 20-h (*A* and *C*) assays. Results are expressed as percentage of specific lysis.

significantly recognized in these assays and, therefore, served as internal specificity controls. E41 was highly recognized by two of four OvTALs tested and one of two BrTALs. The recognition of these peptides by freshly cultured, *in vitro* unstimulated TALs documents the presence of precursor CTLs specific for these epitopes *in vivo,* suggesting *in vivo* priming to these Ags. E39 (FBP, 191–199) appears to be an immunodominant CTL epitope. The overlapping peptides E40 (FBP 247–255) and E41 (FBP 245–253) form a subdominant CTL epitope, because both induced CTL recognition. E41 was better recognized than E40, and its level of recognition was comparable with E39 (19).

FBP Peptide E39 Is a Naturally Processed Ag. To determine whether E39 reconstitutes CTL epitopes that are presented on ovarian tumor cells, cold target inhibition assays were performed. T2 pulsed with E39 (T2–39) were used to block the recognition of TAL populations for the ovarian cancer cell line, SKOV3.A2. T2–39, but not T2–37 or T2-NP, effectively inhibited the tumor lysis by OvTAL-1 (Fig. 2, *A* and *B*) and OvTAL-4 (Fig. 2, *C* and *D*) in 5 h (Fig. 2, *B* and *D*) and 20 h (Fig. 2, *A* and *C*) cytotoxicity assays ($P < 0.05$). The inhibitory effects of T2/E39 increased over time, between 5 and 20 h, suggesting that the recognition of the epitope formed by E39 was not transient or nonspecific. These experiments were repeated, and the results were confirmed (data not shown). These findings suggest that the CTLs specific for the E39 epitope contribute significantly to the recognition of this ovarian cancer cell line. Furthermore, these data demonstrate that FBP-derived peptides are naturally processed Ags. The fact that the levels of lysis were low at 5 h but significantly increased over time suggested that: (*a*) FBP-specific CTLs may be present at low frequency in TALs; and/or (*b*) FBP-specific CTLs represent memory effectors that require restimulation for high expression of CTL function (26).

Induction of Proliferation and Specific IFN-γ Release by FBP Peptides E39 and E41. The results (Figs. 1 and 2) indicate the presence of FBP-specific CTLs in TALs that express low levels of recognition of tumor Ags. This raised the question of whether CTL functions can be amplified. To address this question, we used T2 cells as APCs because they can be exogenously loaded with peptides. Except for low concentrations of IL-2 added 48 h after stimulation, no other costimulatory agents or CTLs supporting cytokines were used to assess the sensitivity of these CTL-TALs to peptide stimulation. Short-term cultured TALs were split into parallel cultures and stimulated with irradiated T2 loaded with either E37, or E39, or E41, or NP as a control. E41 was chosen over E40 as the subdominant epitope to study because of its better recognition by TALs. E40 and E41 differ by only two terminal residues. Because E41 is recognized more frequently and better than E40, we hypothesized that E41 forms a more closely related epitope recognized by TALs than E40. Because T2 are allogeneic to the responders, a certain level of allospecific proliferation and cytokine secretion to T2-NP was expected. For this reason, T2E-37 was used as an additional internal specificity peptide control. One week later, cells in these cultures were counted, and proliferation indexes were determined as compared with the T2-NP- and T2E-37-stimulated cultures. Fig. 3*A* shows the effects of E39 and E41 stimulation on OvTAL-1 and OvTAL-2 numbers. Both peptides induced enhanced TAL proliferation over the level observed with T2-NP in both populations ($P < 0.05$); E39 was superior to E41 in OvTAL-1, whereas E41 was better than E39 in inducing OvTAL-2 proliferation. This suggested differences in precursor frequency and state of activation between the responders in these cultures.

To determine whether FBP peptide stimulation induces cytokine production, we determined the levels of IFN-γ and IL-4. E39 and E41 induced specific IFN-γ release in peptide-stimulated OvTAL. Both E39- and E41-stimulated OvTAL-2 demonstrated peptide-specific IFN-γ production at 24 h (not shown), and the IFN-γ release increased at 48 h (Fig. 3*B*). Similar results were obtained with OvTAL-1. No IL-4 was produced in response to these peptides in either TAL culture (Fig. 3*B*). These findings suggest that the FBP peptides have the ability to activate IFN-γ secretion, which may be relevant for the cytokine-mediated effector pathway. These results also show that IFN-γ production does not parallel OvTAL proliferation. E39 was a weaker stimulator of OvTAL-2 proliferation than E41; however, the IFN-γ levels induced by each peptide were similar.

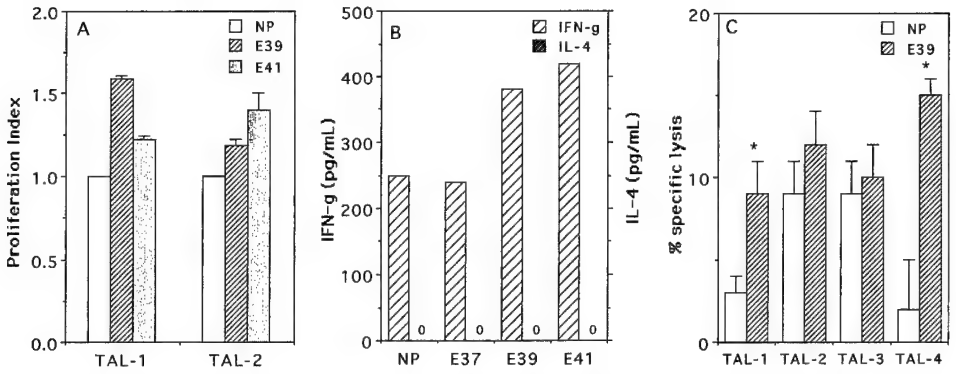

Fig. 3 Stimulation with FBP peptides results in enhanced OvTAL proliferation and IFN-γ release and cytotoxicity. *A,* OvTAL-1 and OvTAL-2 respond differently to E39 and E41. OvTALs were cultured in parallel and stimulated with irradiated T2 loaded with E39, E41, or no peptide (*NP*). Cell counts were performed after 1 week. Results are expressed as proliferation index. *B,* OvTAL-2 demonstrate peptide-specific IFN-γ release. Parallel cultures of OvTAL-2 stimulated with T2 loaded with E37, E39, E41, and NP were replated at 1×10^6 cells/ml in 1 ml and restimulated with T2 and the corresponding peptides E39, E41, E37, or T2-NP. IFN-γ and IL-4 were measured 48 h later. This result is representative of replicated experiments. *C,* enhanced cytotoxicity after FBP peptide stimulation. OvTALs 1–4 were stimulated in parallel with irradiated T2 loaded with E39 or NP. Recognition of E39 was assessed in standard CTL assays after 1 week at an E:T of 10:1. Results are expressed as percentage of specific lysis (*, $P < 0.05$ *versus* NP); *bars,* SE.

Enhanced Cytotoxicity of FBP Peptide-stimulated TALs. One week after primary E39 stimulation, the TAL cultures were evaluated for specific Ag recognition. We used lower E:T ratios (5:1) than in the assays with fresh TALs on the rationale that if there was an increase in specific CTLs compared with the original levels, elicitation of specific recognition should be detected when fewer effectors were used. Two of four TAL populations (OvTAL-1 and OvTAL-4) revealed an increase in lytic activity, on a per cell basis, for E39 compared with parallel cultures stimulated with T2-NP (Fig. 3*C*), whereas OvTAL-3 was resistant. These results show individual related differences in the responsiveness of OvTAL to E39 and E41 when presented on T2 cells. These results indicate a requirement for the relevant peptide for CTL-TAL expansion and expression of the lytic function. OvTAL-2 stimulated with E39 showed weak specificity for E39 (≤25% increase over controls). E41-stimulated OvTAL-2 also exhibited marginal specificity to E41 (38% increase over control T2-E37; data not shown). In terms of amplification of CTL activity, OvTAL-2 and OvTAL-3 were considered nonresponders. OvTAL-2 responded to E39 and E41 with similar high levels of IFN-γ, preferential proliferation to E41 *versus* E39, but poor activation of CTL activity. Because under conditions of allogeneic stimulation, which are expected to amplify an alloreactive response, restimulation of TALs with the self-peptide E39 succeeded in amplifying a CTL response to the tumor, E39 is immunogenic.

Epithelial Tumor Lysis by FBP Peptide-stimulated TALs. In separate experiments, OvTAL-1 and OvTAL-2 stimulated twice by FBP peptide-loaded T2 over 2 weeks were evaluated for recognition of multiple tumor targets at an even lower E:T ratio of (2.5:1) compared with the previous experiments. To establish whether E39 stimulation enhances recognition of SKOV3.A2 (HER-2^{hi}, FBP^{+}), we used as effector OvTAL-1 stimulated with T2-E39 and T2-E41, or with T2-NP and T2-E37 as controls. The results (Fig. 4*A*) show that T2-E39 stimulated OvTAL-1 recognized SKOV3.A2 at significantly higher levels than control (T2-NP/E37)-stimulated OvTAL-1. Of interest, the levels of SKOV3.A2 lysis observed with 1×10^4 effectors in this assay were similar with the levels observed with 8×10^4 unstimulated effectors of the same line (Fig. 2), indicating that E39 stimulation lead to an enrichment in E39-specific effectors. The recognition of FBP-expressing epithelial tumors was verified by using as targets lines expressing different levels of HER-2. OvTAL-1 lysed SKOV3.A2, SW480, and the pancreatic line PAN-1 significantly better than SKBR3.A2 (Fig. 4*B*).

Lysis of these targets appeared not to be dependent on the levels of HER-2. SKOV3.A2 expresses significantly higher levels of HER-2 than SW480 and PAN-1. However, all three lines were recognized at similar levels by OvTAL-1. SKBR3.A2 expresses similar HLA-A2 levels as PAN-1 and SW480. The HLA-A2 levels in these targets are lower than of SKOV3.A2. SKBR3.A2 expresses higher levels of HER-2 than SKOV3.A2. SKBR3.A2 was lysed only marginally by E39-stimulated OvTAL-1. Because all of these tumors express HER-2, one might suggest that this Ag system is being recognized, although one would expect less HER-2 recognition of the lower HER-2-expressing tumor SW480 and higher recognition of SKBR3.A2. This was not seen in these experiments. In Western blotting using MOV18 mAb, we detected very low levels of FBP in SKBR3.A2 cells, compared with SKOV3.A2. Thus, the increase in lysis of SKOV3.A2 *versus* SKBR3.A2 by E39-stimulated OvTAL-1 is supportive of the hypothesis of recognition of E39 or of structurally similar epitope rather than of HER-2.

To verify whether tumor recognition is dependent on stimulating Ags, we tested the same targets for lysis by the weak responder OvTAL-2 restimulated with E39 and E41 or with E37 as control. Results in Fig. 4*C* show that E39 and E41 were weak stimulators of OvTAL-2 cytotoxicity for tumors compared with OvTAL-1. However, OVTAL-2 stimulation with E41 increased recognition of SKOV3.A2, although marginally over the recognition by E37-stimulated OvTAL-2. In contrast, E39 stimulation

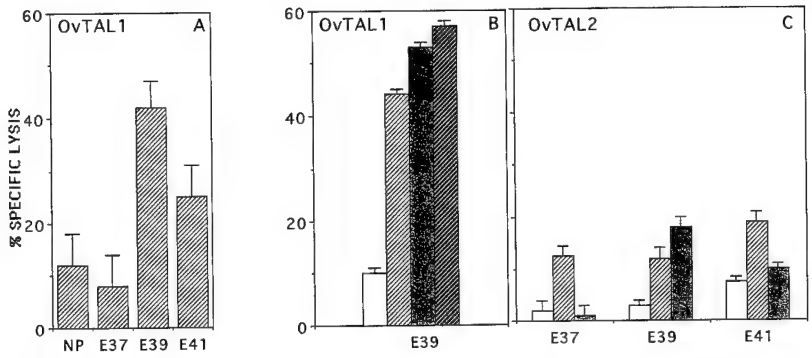

Fig. 4 FBP peptide-stimulated OvTALs recognize tumors of different histologies. *A,* OvTAL-1 was stimulated in parallel with irradiated T2 loaded with E37, E39, E41, or NP twice at weekly intervals and tested at the same E:T ratio (2.5:1) for lysis of SKOV3.A2. *B,* E39-stimulated OvTAL-1 recognize FBP-expressing tumors of different histologies: breast SKBr3.A2 (HLA-$A2^{low}$, HER-2/neu^{high}, $FBP^{\pm}$), □; ovarian SKOv3.A2 (HLA-$A2^{high}$, HER-2/neu^{high}, FBP^{+}), ▨; colon SW480 (HLA-$A2^{low}$, HER-2/neu^{low}, FBP^{+}), ■; and pancreas PAN-1 (HLA-$A2^{low}$, HER-2/neu^{high}, FBP^{+}), ■. *C,* OvTAL-2 stimulated twice at weekly intervals with irradiated T2 loaded with E37, E39, or E41 were tested for recognition of tumor targets expressing FBP. Results are expressed as percentage of specific lysis; *bars,* SE. All TAL-1 cultures were tested in standard 20-h cytotoxicity assays at an E:T ratio of 2.5:1.

increased recognition of SW480 compared with stimulation with E41 or E37. These results support the possibility of differences in epitope presentation by these tumors.

Stimulation of PBMCs from Healthy Donors with DC-E39 Induces E39-specific CTL Activity IFN-γ and IP-10 Production. The results presented above demonstrated that E39 and E41 can stimulate effector functions in OvTAL. However, for preventative vaccination approaches, the ability of a tumor peptide to stimulate CTLs from peripheral T cells of healthy donors is an important factor in considering its immunogenicity. To address whether E39 can activate CTL activity from healthy donor PBMCs, we used an autologous system. In this system, the autologous PBMC-derived DCs were used as APCs. The results of primary stimulation with E39 pulsed DCs (DC-E39) are shown in Fig. 5. We used three different donors to ensure that the results are relevant. The HER-2 peptide E71 was used as specificity control, because it binds HLA-A2 with very low affinity, lower than E39, and it is not recognized by HER-2-specific CTL-TALs (5). Its presence in the assay aimed to balance the exogenous E39 concentration delivered to T2 cells. The results of primary stimulation show an individual-dependent pattern of lytic responses to activation by E39. Donor 1 responded with weak E39-specific CTL activity, donor 2 responded with high levels of E39-specific CTL activity, whereas donor 3 showed no response to E39 in a 5-h CTL assay. The presence of activated E39-specific CTLs was confirmed in donor 3 by retesting the CTL lysis in a 20-h assay. These results indicate that E39 precursors are present in the peripheral blood of healthy donors, and in a fraction of donors, they can be rapidly activated by peptide to exhibit E39-specific lysis.

To determine whether DC peptide stimulation can induce IFN-γ, PBMCs from donor 1 were stimulated in a separate experiment with DC-E39 and DC-E41. DC-NP and DC-E75 (HER-2 peptide) were used as negative and positive controls, respectively. The results (Fig. 6) show that E41 was more potent than E39 in induction of IFN-γ from T cells of this donor within 48 h after primary stimulation. IFN-γ levels secreted in response to E41 were similar to levels induced by E75. The weak IFN-γ

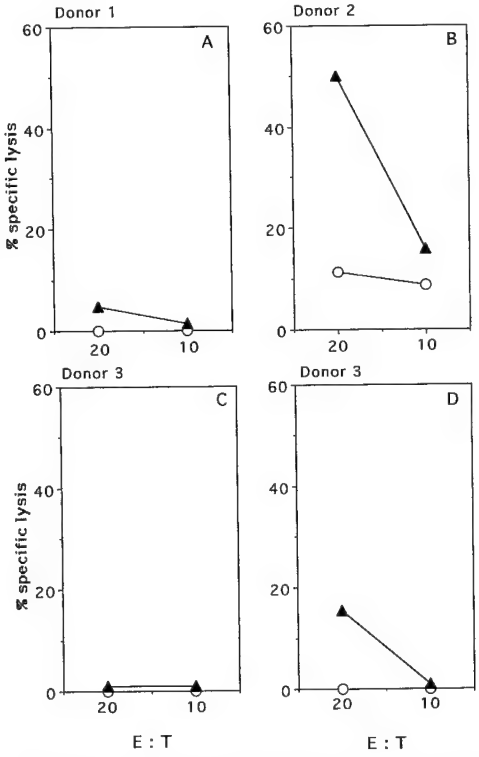

Fig. 5 E39 recognition by primary E39-stimulated PBMCs from three healthy donors. Autologous DCs were used as APCs. T2 cells were pulsed with E39 (▲) or control E71 (○) peptide at 25 μg/ml. Results are from 5-h (*A–C*) and 20-h (*D*) CTL assays. Experimental conditions are as described in "Materials and Methods."

stimulatory ability of E39 was confirmed with another donor (data not shown). IFN-γ production in response to E39 and E41 was not detected within the first 6 h of peptide stimulation, suggesting that activated E39/E41-specific CTLs are not present in this healthy donor.

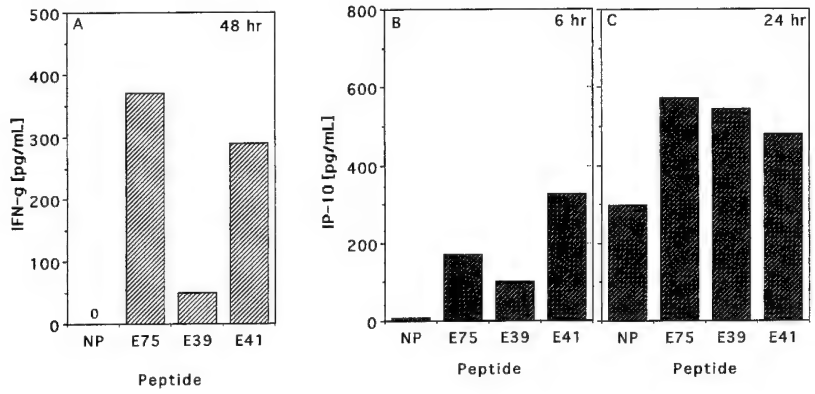

Fig. 6 *A*, E39 and E41 induced IFN-γ from isolated $CD8^+$ cells of healthy donors. Peptides were presented by autologous CD14-derived DCs for 48 h in the absence of IL-2 or IL-12. E75 at the same concentration was used as positive control. *B* and *C*, E39 and E41 at 50 μg/ml rapidly induce IP-10 from isolated $CD8^+$ cells (*B*) or isolated PBMCs from distinct healthy donors. Results indicate pg/ml, 10^6 cells. HER-2 peptide E75 was used as a positive control.

Because different $CD8^+$ functions are sensitive to different levels of Ags, we determined in parallel from the same culture supernatants the presence of the antiangiogenic chemokine IP-10. IP-10 was detected within 6 h of stimulation from this donor in response to both E39 and E41 (Fig. 6*B*). Induction of IP-10 by E39 and E41 was confirmed with a second donor (Fig. 6*C*). There were differences in the levels of response to E39 and E41 between these two donors, suggesting that factors independent of the peptide sequence control the immunogenicity of FBP epitopes. These results indicate that E39 and E41 are immunogenic and can activate distinct effector functions in PBMCs from healthy donors, a property that makes FBP valuable for cancer vaccine development.

DISCUSSION

In this study, we found that freshly cultured ovarian and breast TALs, not previously subjected to Ag stimulation (anti-CD3, autologous tumor) *in vitro,* recognize FBP peptides, and most consistently E39 (FBP, 191–199). Cold target inhibition studies demonstrated that the antigenic peptides E39 and E41 are naturally expressed epitopes on ovarian cancer cells. Together, these data prove that FBP functions as a TAA recognized by CTL-mediated immunity in these cancers. Both immunodominant and subdominant epitopes have been described for the HER-2 protein (4, 5). This concept also holds for FBP, with E39 serving as an immunodominant peptide with the most consistent and highest level of recognition among consecutive TAL populations. E41 appears to be a subdominant epitope with high levels of recognition among some ovarian and breast TAL cultures but not others. These observations are important for understanding the T-cell repertoire responding to a processed tumor (self) Ag. Identification of two CTL epitopes on FBP may proffer a selective advantage for its use for cancer vaccination studies because FBP is expressed in outbred populations. In other systems of immunological disease under $CD8^+$ cell control, shifts in epitope dominance have been described recently and may be favorable or unfavorable predictors of disease outcome (27). Therefore, strategies to enhance immunity to tumors may need to be adjusted based on the presence of individual epitopes (27).

Analysis of FBP peptide immunogenicity indicated that E39 has the ability to activate both major CTL effector functions from TALs, *i.e.,* cytolysis and cytokine (IFN-γ) secretion. The stimulatory effects on cytolysis were evident in two of four OvTALs tested. E39 and E41 peptide-stimulated CTLs were capable of lysing FBP-expressing tumors of different histologies, *i.e.,* ovarian, colon, and pancreas. These tumors have been shown by different groups using biochemical, immunohistochemical, and binding approaches to express FBP (16). E39-stimulated OvTALs not only could recognized allogeneic HLA-$A2^+$ ovarian cancer cells but also lysed other FBP-expressing HLA-$A2^+$ epithelial colon and pancreatic cells. A common feature of E39- and E41-specific TALs was that they induced low levels of lysis in short term (5-h) assay but high levels of lysis in long 20-h assays. We do not know at this time whether this reflects the low density of E39/E41-specific CTLs in TALs or high-density, FBP-specific CTLs that lyse targets using predominantly the Fas-FasL instead of the perforin pathway (28).

In developing an immune response to cancer, the critical test of vaccination strategy is the ability of the Ag to activate primary responses by naïve/resting effectors. This requires significantly stronger signaling by Ags and costimulation than activation of memory responses or restimulation of established effector CTL clones (*i.e.,* from CTL-TALs; Ref. 29). Primary *in vitro* stimulation of T cells from PBMCs of healthy donors with autologous DCs pulsed with E39 resulted in induction of specific recognition of E39 in cytolytic assays, in addition, because it stimulated both IFN-γ and IP-10 secretion. The latter was more sensitive than IFN-γ secretion to FBP. Because IP-10 and other non ELR-CXC chemokines have been implicated in angiostasis and tumor regression (30), our results indicate that FBP may bear a significant immunogenic potential. The fact that IP-10 was detected at a time (6 h) when IFN-γ in culture was not detectable deserves further consideration. Another possibility is that low levels of IFN-γ were absorbed or consumed. Another possibility is that IP-10 induction in $CD8^+$ cells may not require IFN-γ, because it was demonstrated that IFN-γ is not absolutely necessary for *in vivo* IP-10 expression (31). Furthermore, stimulation of OvTALs with DC-E39 resulted in rapid amplification of OvTAL specificity for E39 compared with

control DC-NP stimulation.[4] These results indicate that E39 is immunogenic, *in vitro,* in peptide form when DCs are used as APCs, and activation induced by E39 and E41 may condition the environment for type 1 cytokine response by attracting $CD4^+$ cells of Th1 phenotype (32). This response may protect from Th2 cytokine-induced tolerance.

In contrast with E39, E41 activated IFN-γ production in both PBMCs and TALs, but its stimulatory effects on cytolysis were weak. It should be noted that both E39 and E41 stimulated TAL proliferation. This suggests that E39 and E41 may have distinct effects in activation of different effector functions. The weak IFN-γ response induced by E39 compared with E41 may indicate that memory E39-specific CTLs are present with low frequency in healthy donors (26), but also that E41 has a stronger activating ability of APC/T cells for IFN-γ production. The reasons for the different effects of E39 and E41 in activation of CTL effector functions are under investigation. E41 binding affinity to HLA-A2 is only slightly lower than E39. Furthermore, OvTALs recognized E39 and E41 at similar levels, but E39 specificity was detected more frequently in four of four *versus* two of four TALs, respectively. It is possible that the resistance of certain TALs to stimulation by E41 (and sometimes by E39) reflects the presence in these patients of functionally silenced CTL clones. Thus, E41-specific CTLs may be tolerized more frequently or more extensively. Such CTLs are currently described in studies with conventional Ags, whereas attempts are being made to devise approaches to reactivate their silenced functions (27, 29). The facts that the functional silencing of these clones is attributed to either Ag stimulation in the absence of costimulation (29) or lack of epitope-specific helper $CD4^+$ cells (33), or cytokine (IL-4, IL-10, and transforming growth factor β)-induced tolerance (34) may be relevant to the FBP system.

One of the most promising aspects of epithelial cancer vaccine development is that epithelial tumors share common CTL-recognized epitopes, indicating that a TAA-specific vaccine may be widely applicable. Our results show that E39-stimulated OvTALs lyse ovarian, colon, and pancreatic tumor lines. Because FBP is overexpressed in 90% of ovarian and 50–70% of breast cancers, these results further illustrate the clinical potential for targeting a widely expressed tumor Ag like FBP.

FBP was originally identified independently by three lines of investigation (reviewed in Ref. 19) as: (*a*) the LK26 Ag (16); (*b*) the Ag recognized by MOv18 and MOv19 mAbs (32); and (*c*) the high-affinity FBP from placenta and KB carcinoma cell lines (17). The true extent of FBP expression is still unknown because the mAbs currently used require more complex techniques than simple immunofluorescence staining to detect FBP (18). Because of some intrinsic differences between the LK26, MOv18, and MOv19 mAbs, different levels of FBP expression on some tumors have been reported. There is general agreement that only low levels of FBP expression exist in some normal tissues such as choroid plexus, lung, thyroid, kidney, and sweat glands.

High levels of FBP are preferentially expressed in a wide range of cancerous tissue (18). The reason for this differential expression is unknown, but one hypothesis involves the upregulation of the folate receptor to compensate for the loss of the alternative folate processing pathway involving the *tetrahydrofolate* reductase gene, which is frequently deleted in cancer cells (35, 36). The highest levels of expression of FBP have been found in ovarian carcinomas, with >90% of all ovarian carcinomas expressing elevated levels of this protein. The levels of overexpression have been shown to be >20 fold that of normal tissue routinely and reported to be as high as 80–90-fold in one study (18, 19). The extent of FBP expression in other tumors is probably underestimated, as alluded to above; however, multiple tumor types have been shown to overexpress the LK26/FBP Ag, including 20–50% of colorectal, breast, lung, and renal cell carcinomas, as well as many other tumor types (19).

Soluble peptide vaccines with a single peptide CTL epitope and immunoadjuvant are currently in trials for both melanoma (37) and the E75, HER-2 peptide (38). The complete response data are not yet available. Early results indicate that in the use of tumor peptide E75 as immunogen with incomplete Freund or granulocyte/macrophage-colony stimulating factor as adjuvant may not lead to tumor-specific CTL and clinical responses (38, 39). Because FBP peptides are recognized by freshly isolated OvTALs that are activated *in vivo* and lyse autologous tumors, development of approaches for *in vitro* and *in vivo* activation of CTLs by those peptides becomes a key issue. Immunogenicity for CTL induction is dependent on the Ag sequence and the delivery system to APCs. Although some conventional or viral Ag (*e.g.,* influenza) can induce CTLs by helper-independent, costimulation-independent mechanisms (33), the majority of known tumor peptides require costimulatory signals (from APC molecules or cytokines; Ref. 40) or Ag modification to induce CTL differentiation (29). The fact that E39 can activate both TALs and PBMCs suggests that E39 is a potentially immunogenic epitope for pCTL activation.

We are currently investigating the dominant and subdominant epitopes from both HER-2/neu (E75, GP2, and C85) and FBP (E39 and E41) in parallel to determine the immunogenicity of each of these epitopes to induce primary CTLs with tumor killing ability and the requirement for proinflammatory cytokines, such as IL-12, in activating APCs or reversing tolerance. We are also investigating the possibility of immune-gene therapy for these types of epitopes by delivering the minimal CTL epitope together with the endoplasmic reticulum translocation sequence using vaccinia vectors to the APCs and proteasome inhibitors to enhance targeting of the epitope precursor protein to the MHC-I processing and presentation pathway.

As effective delivery systems are developed, the key to a successful epithelial cancer vaccine still depends on the discovery of CTL-recognized TAAs to be targeted with the vaccine. In conclusion, FBP serves as an endogenous source of CTL-recognized and CTL-stimulatory epitopes, which are naturally expressed on a large number of epithelial tumors. Because FBP is overexpressed with high frequency, FBP peptides E39 and E41 may serve as the basis of a widely applicable epithelial cancer vaccine. Clinically, these findings would support the development of a polyspecific vaccine composed of dominant and subdominant peptides from either a single or multiple TAAs.

[4] D-K. Kim and C. G. Ioannides, manuscript in preparation.

ACKNOWLEDGMENTS

We thank Drs. Georgia Thomas and Catherine A. O'Brian for critical reading of the manuscript and helpful comments.

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Cancer Immunol Immunother (1999) 48:401–410

ORIGINAL ARTICLE

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HER-2/*neu* peptide specificity in the recognition of HLA-A2 by natural killer cells

Received: 16 March 1999 / Accepted: 3 June 1999

Abstract Although natural killer (NK) cells have been described as non-MHC-restricted, new evidence suggests that NK activity can be either up- or down-regulated after interaction with the peptide–MHC-class-I complex expressed on target cells. However, the epitope(s) recognized by NK cells have remained ill-defined. We investigated NK cell recognition of synthetic peptides representing a portion of a self-protein encoded by the HER-2/*neu* (HER-2) proto-oncogene and presented by HLA-A2. HER-2 nonapeptides C85, E89, and E75 were found partially to protect T2 targets from lysis by freshly isolated and interleukin-2(IL-2)-activated NK cells (either HLA-$A2^+$ or $A2^-$). This inhibition was not solely due to changes in the level of HLA-A2 expression or conformation of serological HLA-A2 epitopes. Using single-amino-acid variants at position 1 (P1) of two HER-2 peptides, we observed that protection of targets was dependent on the sequence and the side-chain. These results suggest similarities in the mechanism of target recognition by NK and T cells. This information may be important for understanding the mechanisms of tumor escape from immunosurveillance and could help explain the aggressiveness of HER-2-overexpressing tumor cells.

Key words Natural killer cells · HER-2/*neu* · Peptides · MHC · Tumor immunity

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Introduction

Natural killer (NK) cells are thought to play an important role in the elimination of virus-infected cells and cancer cells [5, 26, 39]. Although target-cell killing by NK cells has traditionally been described as non-MHC-restricted, interaction of NK-cell-inhibitory receptors with MHC class I molecules often leads to a down-regulation of NK cytolytic function in proportion to the level of MHC class I expression on the targets [20, 27, 40, 42]. Recent reports also indicated that single amino acid mutations within the peptide-binding groove of the MHC molecule can affect target cell sensitivity to lysis, suggesting that NK cells recognize different conformations induced by peptides bound in the MHC class I pockets [19, 37]. This hypothesis has been supported by observations that external loading of target cells with either self or foreign peptides can enhance or inhibit sensitivity to NK-mediated lysis in a peptide-specific manner independent of the level of MHC class I up-regulation [7, 25, 28, 38]. However, the basis for peptide specificity in the induction of lysis or protection is unknown. Further analysis of the mechanism of NK recognition of peptides may provide an important insight into the function of NK cell specificity for tumor cells.

The HER-2/*neu* (HER-2) proto-oncogene product is overexpressed in a variety of human cancers including breast, ovarian, colon, lung, and stomach, and its overexpression by breast and ovarian cancers has been shown to correlate with earlier relapse and a worse prognosis [36]. Since it has been reported that HER-2 overexpression also correlates with decreased NK cell activity [41], we wanted to determine if HER-2 peptides are directly involved in NK cell inhibition. Therefore, we used HER-2 peptides recognized by cytotoxic T lymphocytes (CTL) as targets, where the question of the sequence specificity in NK recognition can be addressed. In this report we investigated the ability of freshly isolated and in vitro interleukin-2(IL-2)-activated NK cells to recognize self-HER-2 peptides that bind to the HLA-

A2 molecule with variable affinities and induce conformational changes in the α1 and α2 domains. We found that these peptides decreased NK-mediated lysis of T2 cells, and the ability to inhibit lysis depended more upon peptide sequence than the ability to up-regulate or induce conformational changes of HLA-A2 on the target cells. Interestingly, the peptide that induced the most HLA-A2 up-regulation and conformational changes inhibited lysis least. Furthermore, targets pulsed with HER-2 peptide variants containing amino acid substitutions at position 1 (P1) showed either side-chain-dependent protection or increased sensitivity to NK-mediated lysis. Again, increased levels of lysis inhibition among the peptides did not correlate with increased levels of expression of HLA-A2, as detected by the W6/32 mAb specific for a monomorphic MHC I epitope (α3 domain), and lysis inhibition did not correlate with conformational changes of HLA-A2 detected by the MA2.1 mAb (α1 domain). Peptides that induced the most change in expression and conformation of HLA-A2 were often less effective at inhibiting lysis. However, the enhanced sensitivity to NK lysis seen with one peptide was paralleled by changes in the conformational epitope recognized by the BB7.2 mAb (α2 domain). These results indicate an important effect of changes in peptide sequence at position 1, and provide further evidence that the mechanism of NK target recognition has some similarity to that of T cells in that it is determined by interactions of peptide side-chains with NK receptors. These findings may also be helpful in explaining why cancer patients with tumors overexpressing HER-2 have a worse prognosis.

Materials and methods

Target cells

The T2 line has been described previously [15] and was a generous gift from Dr. Peter Cresswell (Yale Univeristy School of Medicine, New Haven, Conn.). The B cell line C1R:A2, an HLA-A2-gene-transfected derivative of C1R, was a gift from Dr. William Biddision (National Institute of Neurological Disorders, Bethesda, Md.). C1R:A2 cells were transfected with the plasmid pCMV.HER-2 encoding a full-length HER-2 cDNA (the kind gift of Dr. Mien-Chie Hung, Department of Tumor Biology, M.D. Anderson Cancer Center). C1R:A2:HER-2 transfectants were selected by resistance to hygromycin B by co-transfection of SV2.Hygro plasmids (ATCC, Rockville, Md.).

Effector cells

Peripheral blood buffy coats of normal donors were purchased from a local blood center, and mononuclear cells (PBMC) prepared by Ficoll-Hypaque gradient separation [24]. NK cells were enriched to high purity by negative selection using a MACS NK Isolation Kit (Miltenyi Biotec, Auburn, Calilf.). In brief, PBMC were incubated for 15 min at 4 °C with a cocktail of monoclonal antibodies (mAb) recognizing CD3, CD4, CD19, and CD33, washed, and then incubated for an additional 15 min with colloidal superparamagnetic microbead-labelled antibody reacting to the primary antibodies (Beckton Dickinson, Mountain View, Calif.). The cells were then passed twice through an iron-wool column placed within a strong magnetic field, and the nonadherent cells collected. The effluent population was routinely 91.7%–98.2% $CD56^+$, $CD3^-$ NK cells, 0.1%–1.4% $CD56^+$, $CD3^+$ T cells, and 0.2%–1.3% $CD56^-$, $CD3^+$ T cells as determined by two-color flow cytometry [35].

For IL-2 activation, NK cells were cultured for 5–7 days in RPMI-1640 medium supplemented with 10 mM HEPES buffer, 10% human AB serum, antibiotics, 2 mM glutamine, 2 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μM 2-mercaptoethanol (complete RPMI medium), and 500 U/ml highly purified human recombinant rIL-2 (18×10^6 IU/mg; Cetus Corp., Emeryville, Calif.). The NK cell line, NKL (kindly provided by Dr. M.J. Robertson, Dana Farber Cancer Institute, Boston, Mass.) was obtained from peripheral blood of a patient with a $CD3^-$, $CD16^+$, $CD56^+$ large granular lymphoproliferative disorder [18]. These cells were maintained in culture in complete RPMI medium supplemented with 30 U/ml IL-2.

In some experiments, the CTL line 41 (CTL-41) was used as a source of effectors. This line was developed by repeated in vitro stimulation of $HLA\text{-}A2^+$ peripheral blood mononuclear cells from a healthy donor with peptide C84: HER-2 (971–979 V) and a longer peptide C43: HER-2 (968–981) [12]. For these studies, CTL-41 were maintained in culture with monthly restimulation with 10 μg/ml C84 peptide and autologous or allogeneic $HLA\text{-}A2^+$ PBMC. The CTL used as effectors were selected on mAb-coated plates (AIS Micro CELLector, Applied Immune Sciences, Menlo Park, Calif.), and were $CD3^+$, $CD4^-$, $CD8^+$. Clones were isolated from the CTL-41 line, as previously described [14].

Synthetic peptides

Synthetic peptides corresponding to sequences in HER-2: E75 (369–377), E89 (851–859), C85 (971–979), and recognized by ovarian tumor-specific CTL, have been reported previously [12–14]. The amino acid sequences of these peptides are shown in Table 1. Variants of the C85 peptide substituted at P1 are designated as G1, F1, T1, and K1 [12]. The E75 peptide substituted at P is designated as peptide F41. The synthetic peptides used in this study were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center, purified to 92%–95% by HPLC, and dissolved in phosphate-buffered saline (PBS) at a stock concentration of 1 mg/ml.

Table 1 Sequences of synthetic HER-2 peptides

Code	Position	1	2	3	4	5	6	7	8	9
E75	369–377	K	I	F	G	S	L	A	F	L
F41		G	–	–	–	–	–	–	–	–
E89	851–859	V	L	V	K	S	P	N	H	V
C85	971–979	E	L	V	S	E	F	S	R	M
G1		G	–	–	–	–	–	–	–	–
F1		F	–	–	–	–	–	–	–	–
T1		T	–	–	–	–	–	–	–	–
K1		K	–	–	–	–	–	–	–	–
C84		E	–	–	–	–	–	–	–	U

Cytotoxicity assay

The ^{51}Cr release assay has been described in detail previously [24]. For peptide-pulsing experiments, ^{51}Cr-labelled T2 cells were dispensed into 96-well microtiter plates and preincubated for 2 h in serum-free RPMI medium, to which was added either 10 µl peptide (100 µg/ml final concentration), or an equivalent volume of PBS as a control. Effector cells, suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum, were then added in various E:T ratios (ranging from 40:1 to 1:1), and the culture supernatants were tested for chromium release after 4–5 h of culture. Each experimental condition was tested in triplicate. Results are expressed as the percentage specific lysis according to the formula $(E - S)/(M - S) \times 100$, where E is the radioactivity (cpm) of experimental wells containing both effectors and targets, S is the spontaneous release of ^{51}Cr from targets incubated in medium (with and without peptide), and M represents the radioactivity for targets incubated with 0.2% Triton X-100 (maximum release). In some experiments the cytotoxicity was expressed as lytic units (LU), where 1 LU is the number of effector cells required for lysis of 30% of the target cells [35]; when this calculation is used, the results are expressed as $LU_{30}/10^6$ effector cells.

In studies designed to analyze the sensitivity of HER-2-gene-transfected cell lines to lysis, NK or CTL effector cells were incubated for 4–5 h with ^{51}Cr-labelled C1R:A2:HER-2-transfected target cells at effector-to-target ratios (E:T) ranging from 12:1 to 50:1

Flow-cytometric analysis

Expression of HLA-A2 on T2 target cells was evaluated by flow cytometry, using BB7.2, MA2.1 and W6/32 mAb. W6/32 mAb (Dako, Dakopatts, Denmark) recognizes a monomorphic epitope common to HLA-A, -B, and -C. The anti-HLA-A2 mAb, BB7.2 (mouse IgG2b) and MA2.1 (mouse IgG1) were obtained from the American Type Culture Collection (ATCC). Other antibodies used in this study included anti-CD11a, anti-CD18, anti-CD58, and anti-CD56 (Beckton-Dickinson, Mountain View, Calif.); and Ab2, reacting with the extracellular domain of HER-2 protein (Oncogene Science, Uniondale, New York). Briefly, 5×10^5 cells were incubated for 30 min at 4 °C with primary antibody (or an isotype control antibody nonreactive with human cells), washed, and then incubated for an additional 30 min with fluorescein-isothiocyanate-conjugated goat anti-(mouse Ig). Flow-cytometric analyses were performed on 5000 gated events/sample, using a FACScan flow cytometer (Becton-Dickinson, Mountain View, Calif.) and Consort 30 software.

To analyze the effect of peptide pulsing on HLA-A2 expression, T2 cells were incubated for 2 h at 37 °C with 10–100 µg/ml peptide (or PBS alone as a control), prior to labelling with the primary mAb. All cells tested were positive for HLA-A2 expression; data are reported as the mean channel fluorescence, indicative of the channel number corresponding to the average peak of fluorescence [6, 31, 35].

Statistical analysis

The data were analyzed statistically using Prism 2.01 software (GraphPad Prism for Scientists, Sorrento, Calif.). Multiple groups were compared by the Newman Keuls one-way analysis of variance. When only two groups were compared, Student's t-test was used. Differences were considered significant when P was less than 0.05.

Results

HER-2 peptides inhibit NK-mediated lysis of T2 cells

In the first series of experiments, we investigated the effects of HER-2 self-peptides on the sensitivity of T2 target cells to lysis by NK cells. The T2 cells have a defect in TAP (transporter-associated with antigen presentation) proteins and display "empty" HLA-A2 molecules [33] that can be loaded exogenously with peptides having the proper anchors for binding to HLA-A2, i.e. L/M/I/V (P2) and V/L/M/I (P9). For our studies we used three different synthetic nonapeptides of HER-2 that display these HLA-A2 anchors: E75, E89 and C85 (the amino acid sequences of these peptides are shown in Table 1). These peptides were previously found to reconstitute recognition of $CD8^+$, $CD4^-$ CTL lines derived from ovarian tumor-associated lymphocytes [12], suggesting that HER-2 is naturally processed into identical or similar peptides presented by HLA-A2 on tumor cells.

The T2 targets were pulsed with peptides at a concentration of 100 µg/ml prior to addition of effector cells. In agreement with others [32], we observed that untreated T2 targets were sensitive to lysis by freshly isolated and IL-2-activated peripheral blood NK cells from all healthy donors tested (Fig. 1). However, T2 cells pulsed with HER-2 peptides were significantly protected from killing by unstimulated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1A, B). These peptides also protected T2 targets from lysis by IL-2-activated HLA-$A2^+$ and HLA-$A2^-$ NK cells (Fig. 1C, D). The results from four representative donors of eight tested are shown in Fig. 1. The reduction in lysis of peptide-pulsed

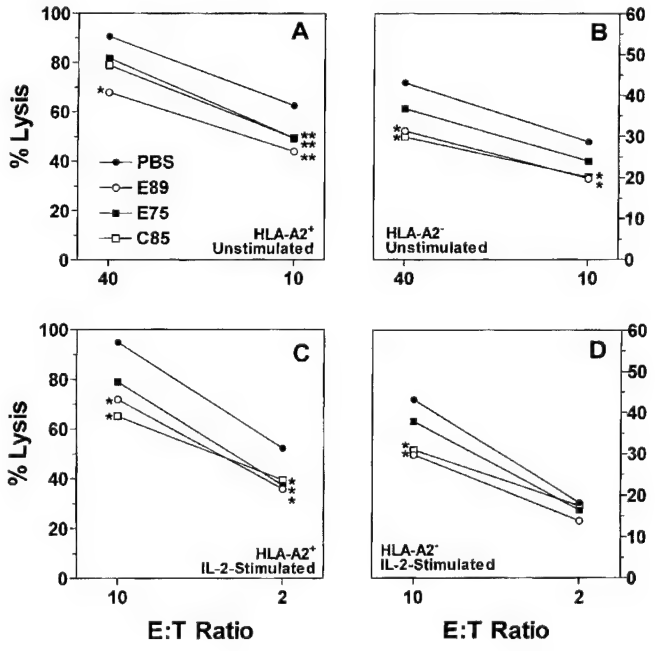

Fig. 1A–D Recognition of HER-2 peptide-pulsed T2 cells by natural killer (NK) cells. Magnetically sorted NK cells from four healthy donors (one per panel) were tested for lytic activity against HER-2-peptide-pulsed T2 cells in a ^{51}Cr-release assay. The NK cells were (**A**) unstimulated HLA-$A2^+$, (**B**) unstimulated HLA-$A2^-$, (**C**) interleukin-2(*IL-2*)-stimulated HLA-$A2^+$, and (**D**) IL-2-stimulated HLA-$A2^-$. Significant inhibition of lysis: $^*P < 0.05$ compared to phosphate-buffered saline (*PBS*) control; $^{**}P < 0.01$ compared to PBS control

target cells was consistently observed at multiple E:T ratios and ranged from 15% to 30%.

Because sensitivity of target cells to NK-mediated lysis has been shown to be inversely related to the levels of MHC class I expression [40], we next determined whether the resistance of HER-2-pulsed T2 targets to lysis by NK cells was associated with an increase in MHC class I molecules caused by peptide-induced stabilization [6]. As shown in Fig. 2, T2 cells incubated with HER-2 peptides displayed an increase in the relative density of surface HLA class I molecules as detected by the HLA-A, -B, -C-specific W6/32 mAb; this was observed as an increase in the fluorescence intensity of mAb-labelled peptide-pulsed T2 cells compared to controls. However, there were marked differences among the peptides in the relative density of class I molecules induced, with an approximately twofold and threefold increase caused by E89 and E75 respectively. The C85 peptide only slightly increased MHC class I expression. Despite these large differences in MHC class I expression, the level of protection afforded by E89 and C85 peptides was comparable. Although E75 increased MHC expression the most, it was consistently least effective at inhibiting target lysis (Fig. 1). These results indicate that the increased resistance of T2 to lysis by NK cells induced by C85 was not simply caused by up-regulation of MHC class I molecules.

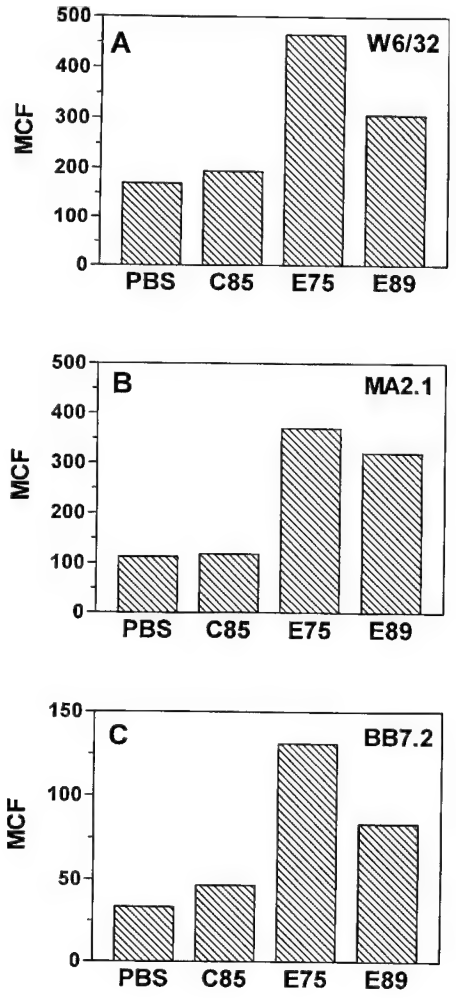

Fig. 2A–C Up-regulation of HLA-A2 expression by HER-2 peptides. T2 cells pulsed with or without (PBS control) HER-2 peptides, were analyzed by flow cytometry for expression of epitopes recognized by the W6/32, MA2.1, and BB7.2 mAb. *Bars* the mean channel fluorescence (*MCF*) value, i.e., the channel corresponding to the mean fluorescence intensity of positively stained cells

To determine if peptide-induced protection was related to changes in the conformation of HLA-A2 molecules, we also analyzed peptide-pulsed and control T2 cells for expression of conformational epitopes recognized by BB7.2 and MA2.1 mAb. The epitope recognized by the BB7.2 mAb is located on the N-terminal loop of the α2 domain (including W108) of HLA-A2, in an area not expected to contact the peptide directly [34]. MA2.1 mAb reacts with the α1 domain of HLA-A2 at residues 64–68, which border the A and B pockets of the peptide-binding groove; mutations of HLA-A2 in this area have been reported to affect T cell recognition significantly [16, 34]. The results in Fig. 2 indicate that the decrease in the sensitivity of T2 to lysis by NK cells, after pulsing with a particular peptide, was not proportional to the increase in the expression of either of these conformational epitopes. On the contrary, while MA2.1 and BB7.2 epitopes were expressed at approximately threefold higher levels on E75-pulsed T2 cells compared to untreated or C85-pulsed targets, the protection induced by E75 was, in most cases, less than that of the other peptides (Fig. 1).

Recognition of peptide variants by NK cells

Crystallography studies have shown that the N-terminal (P1) residue of peptides binds within the A pocket of the MHC molecule, and that the nature of the side-chain of this residue affects peptide binding to HLA-A2 [3]. To address the question of whether a single amino acid substitution at P1 would alter the ability of a HER-2 peptide to protect targets from NK-mediated lysis, we created a series of C85 variants by replacing the glutanic acid at P1 with lysine (variant K1), glycine (variant G1), threonine (variant T1) or phenylalanine (variant F1) (Table 1). These peptide variants do not have changes in the dominant anchors for HLA-A2 at P2 (L/M/I/V) and P9 (V/L/M/I), so they should still bind to HLA-A2. Using a peptide concentration that was protective for C85, we tested the ability of the peptide variants to protect T2 cells from NK-mediated lysis (Fig. 3A). Significant differences between the peptides were observed regarding their ability to affect T2 lysis. Specifically, we found that the K1 variant was as effective as the natural C85 peptide in protecting T2 cells from lysis, while the F1 and G1 variants did not significantly inhibit T2 lysis compared to control T2 cells treated with PBS. In contrast, T2 targets pulsed with the T1 variant not

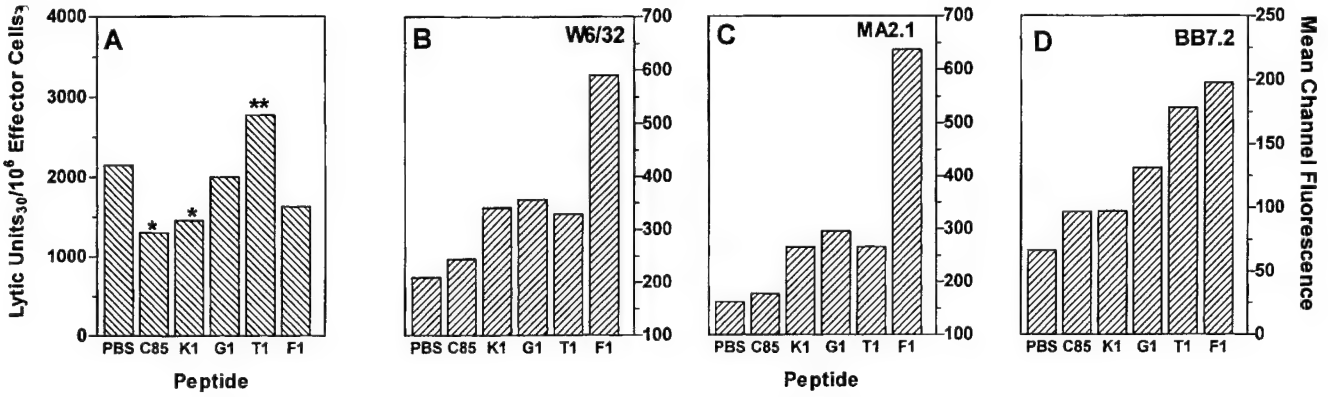

Fig. 3A–D Recognition of HER-2 peptide variants by NK cells. **A** Immunomagnetically isolated HLA-A2$^+$ NK cells were tested for cytolytic activity against T2 targets pulsed with 100 μg/ml C85 and C85 variants, and the results from four separate donors were averaged. Data are expressed as lytic units as described in Materials and methods. * There was significant protection of T2 after pulsing with C85 (E1) or the K1 variant ($P < 0.05$ compared to PBS-treated control T2 targets). ** The T1 variant caused significant enhancement of T2 lysis compared to both C85-pulsed and control targets ($P < 0.05$). **B–D** Three different mAb (W6/32, MA2.1, and BB7.2) were used to detect MHC class I expression by T2 cells pulsed with the same peptides used in **A**

only were not protected, but instead were even more susceptible to lysis by NK cells than were PBS-treated controls. On the basis of a comparison of cytotoxicity (LU), they were also twofold more susceptible to NK lysis than C85-pulsed T2.

All of these C85 variants up-regulated and stabilized MHC class I expression as detected by the W6/32 mAb, albeit to different degrees (Fig. 3B). The conformational epitopes recognized by MA2.1 and BB7.2 mAb were also up-regulated when compared to PBS-treated T2 targets (Fig. 3C, D). The levels of expression of W6/32 and MA2.1 HLA-A2 epitopes on T2 cells pulsed with K1, G1, and T1 variants were similar, and they were higher than the levels induced by C85. The F1 variant induced a twofold higher increase in these epitopes relative to the other variants. The T1 and F1 variants induced the highest levels of BB7.2 epitope expression among the variants tested.

When the levels of expression of MHC class I and the BB7.2 and MA2.1 conformational epitopes were compared to protection from lysis, the ability of a particular peptide to down-regulate target sensitivity to NK lysis was not directly proportional to the increase in the level of HLA-A2 expression. For example, neither the G1 nor F1 variant was significantly protective, even though F1 induced a substantially higher expression of W6/32, MA2.1, and BB7.1 epitopes. Furthermore, compared to C85, the T1 peptide enhanced the susceptibility of T2 cells to NK-mediated lysis, even though this variant induced higher levels of HLA-A2 expression and conformational changes. The K1 variant was as protective as C85, but induced higher levels of MHC class I than did the natural peptide, as detected by the W6/32 and MA2.1 mAb. Again, these data support the observation that increased MHC class I does not always correlate with enhanced resistance to lysis by NK cells.

The results in Fig. 3 show a significant role for the P1 residue side-chain in NK inhibition. Protective peptides in this study (C85 and K1) have charged side-chains, while the nonprotective peptides (F1 and G1) have nonpolar side-chains. T1, which enhanced NK sensitivity, has a hydroxyl group. To confirm that the NK inhibition is dependent on the side-chain of the amino acid at P1, we investigated NK recognition of T2 cells pulsed with the weakly NK protective peptide E75 and its P1 variant, F41. In the latter variant, the lysine at P1 in the natural peptide is substituted with glycine, which lacks a side-chain (Table 1). This K → G change at P1 of E75 is identical to the change between the K1 and G1 variants of C85 described above. Thus F41 was expected to be less protective than E75. We observed that both E75 and F41 showed a similar concentration-dependent ability to up-regulate HLA-A2 expression on T2 cells: for example, at 100 μg/ml, the mean channel fluorescence for the expression of the BB7.2 epitope was approximately threefold higher for both E75-pulsed and F41-pulsed targets than for the controls (data not shown). Both peptides also protected T2 targets from lysis by the NK cell line (NKL) when used at concentrations of 10–100 μg/ml (Fig. 4A). However, at 200 μg/ml, E75 (charged P1 side chain) was significantly more protective ($P < 0.001$) against NK lysis than was F41 (nonpolar P1 side-chain), supporting the conclusion from the previous experiment.

To rule out the possibility that the HER-2 peptides were stabilizing non-HLA-A2 MHC or nonclassical MHC molecules that could mediate a decrease in NK sensitivity [1, 2], E75-pulsed T2 targets were treated with either the HLA-A2-specific MA2.1 mAb or control antibody before lysis by the NK cell line was assessed. As seen in Fig. 4B, HER-2-peptide-induced inhibition of lysis was most likely mediated directly through interaction with HLA-A2, because MA2.1 antibody completely blocked the inhibition of T2 lysis at 100 μg/ml E75 ($P < 0.05$) and significantly blocked the higher inhibition at 200 μg/ml E75 ($P < 0.01$).

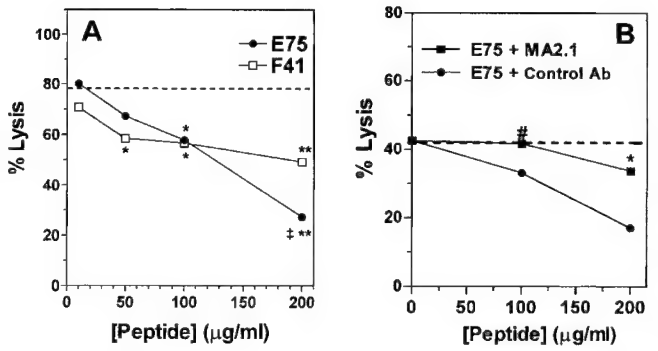

Fig. 4A, B Protection of T2 targets by HER-2 peptides is concentration-dependent and HLA-A2-dependent. T2 cells pulsed with E75 or its variant, F41, were tested for susceptibility to lysis by the HLA-A2$^+$ NK cell line, NKL, at an E:T ratio of 40:1. - - - - The percentage lysis of PBS-treated control T2 targets (78% for **A** and 42% for **B**). **A** Significant protection from lysis: * $P < 0.01$ compared to PBS-treated control; ** $P < 0.001$ compared to PBS control; ‡ E75 inhibited lysis significantly better than F41 at 200 μg/ml ($P < 0.001$). **B** E75-pulsed T2 targets were incubated with HLA-A2-specific blocking antibody (MA2.1) or control antibody. # MA2.1 completely blocked the protection from lysis at 100 μg/ml E75 ($P < 0.05$). * Significant blocking of the protection from lysis also occurred at 200 μg/ml E75 ($P < 0.01$)

Recognition of HER-2-transfected C1R:A2 cells by NK cells

Under physiological conditions, NK cells interact with HLA-A2 molecules presenting peptides processed endogenously. Therefore, it was of interest to determine if endogenously processed HER-2 peptides could also protect target cells from lysis by NK cells. We approached this question using C1R:A2 cells transfected with the HER-2 gene (C1R:A2:HER-2$^+$ cells). C1R:A2:HER-2$^+$ cells were cloned by stringent limiting dilution, and three clones (HER-2:G, HER-2:B, and HER-2:L), expressing different levels of surface HER-2, were tested for sensitivity to lysis by CTL and NK cells. CTL clones (CD3$^+$, CD8$^+$, CD4$^-$) were developed by in vitro stimulation of HLA-A2$^+$ mononuclear cells from a healthy donor with C84, a P9-substituted (M → V) C85 peptide [12]. As shown in Fig. 5, two clones (2C2 and 2C3) recognized the C1R:A2:HER-2:L clone but not C1R:A2 cells lacking HER-2 gene expression, suggesting that an epitope similar to C85 was presented by C1R:A2:HER-2$^+$ cells. However, the HER-2$^+$ clones were more resistant to lysis by IL-2-stimulated NK cells than were the nontransfected

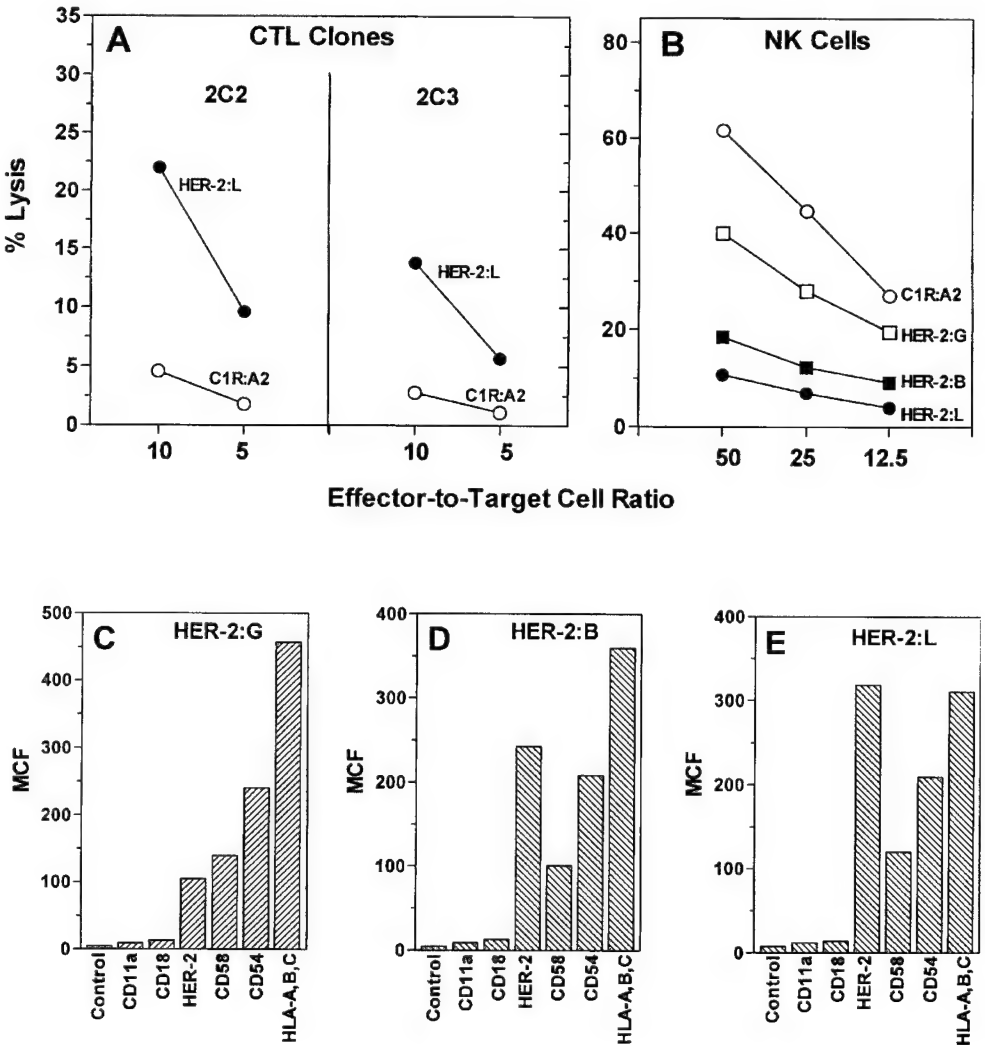

Fig. 5A–E Lysis of the HER-2 gene-transfected target cells by cytotoxic T lymphocytes (CTL) and NK cells. **A, B** Nontransfected and HER-2-transfected CIR:A2 cells (HER-2:G, HER-2:B, and HER-2:L) were tested for susceptibility to lysis by two C85-specific HLA-A2$^+$ CTL clones (2C2 and 2C3) and magnetically sorted, IL-2-activated HLA-A2$^+$ NK cells. The HER-2 transfectants were clones chosen for their high (HER-2:L), medium (HER-2:B), or low (HER-2:G) expression of HER-2. **C, D, E** Surface MHC class I, HER-2, LFA-1a (CD11a), LFA-1b (CD18), LFA-3 (CD58), and ICAM-I (CD54) expression by C1R:A2 and HER-2 transfectants was analyzed by flow cytometry

targets. Furthermore, the sensitivity of the clones to lysis by NK cells varied inversely with the density of HER-2 expression; i.e. the sensitivity of the transfectants to lysis ranked G > B > L, while HER-2 expression ranked L > B > G (Fig. 5).

The HER-2:G targets were most sensitive to NK lysis even though they expressed a higher density of HLA-A2 than did HER-2:B and HER-2:L. Additional phenotypic analyses of these clones revealed that they all expressed only very low levels of CD18 (LFA-1β) and CD11a (LFA-1α), while CD54 (ICAM-1) and CD58 (LFA-3) were expressed at similar levels among the cloned transfectants (Fig. 5C–E). Therefore, there was no correlation between adhesion molecule expression and the sensitivity of HER-2 transfectants to lysis by NK cells. Our data suggest that quantitative and qualititative changes in the composition of the naturally processed HER-2 peptides presented by MHC, rather than alterations in the expression of MHC class I or adhesion molecules, are responsible for the protective effects of HER-2.

Discussion

In this report we present novel evidence that HLA-A2-binding HER-2 peptides, known to form CTL epitopes, can protect targets from lysis by NK cells. This protection was found to be dependent upon (a) peptide concentration, requiring pulsing with peptides at 50–100 μg/ml; (b) peptide sequence, since single amino-acid substitutions could significantly alter the status of target susceptibility; and (c) side-chain charge, with charged side-chains at position 1 generally inducing more protection from NK lysis than uncharged side-chains. In support of previous studies by others [2, 25, 26, 28, 43], this indicates that NK cells recognizing peptide-MHC complexes display a high degree of target specificity. These findings also suggest that CTL epitopes on tumor cells may block NK lysis, a mechanism that may have implications for tumor survival in the absence of CTL. An increase in the relative ability of a peptide to inhibit lysis was, in most cases, not associated with increased expression of HLA-A2 on T2 target cells, or with conformational changes of HLA-A2 detected by BB7.2 and MA2.1, suggesting that these serological epitopes are not solely responsible for inhibition of NK function.

HLA-A2 conformational changes were often seen on targets that were most sensitive to lysis in this study. For example, increased staining with the BB7.2 mAb was associated with enhanced lysis in the case of the T1 peptide and decreased protection from lysis for E75. One possible explanation for the enhanced sensitivity to lysis of targets bearing HLA-A2 conformational changes could be that, although HLA-A2 expression inhibits lysis, it can only do so if the conformation is not altered by the peptide. However, the full explanation is probably more complex, because E89 induced a fair amount of HLA-A2 conformational changes (both MA2.1 and BB7.2) yet inhibited lysis as effectively as C85, a peptide that did not induce such changes in HLA-A2. One alternative explanation for the enhanced sensitivity to lysis caused by T1 is the hydroxylated side-chain (tyrosine) at P1, which may have decreased the recognition of HLA-A2–peptide by an inhibitory NK receptor. Further experiments are necessary to elucidate this mechanism.

In agreement with previous studies, the peptide concentrations required to induce a significant NK-protective effect were higher than the concentrations required to sensitize T2 cells to CTL effectors from breast and ovarian cancer patients [12, 14]. This may indicate that these effects are only relevant in vitro. However, recent studies on peptide binding to HLA-A2 molecules indicate that, during 4–6 h of incubation, the number of class I MHC complexes formed with similar amounts of exogenously added peptides is in the range of 10^3–10^4, which is consistent with the level of expression of a number of endogenous peptides [17]. Therefore our results should be relevant to certain pathological conditions, such as viral infections and cancer, where large amounts of viral or tumor peptides are processed and presented by MHC class I. The observation that NK cells were less effective in lysis of C1R:A2 cells expressing high levels of HER-2, than of those expressing lower levels, is suggestive of this possibility. Thus, protection from NK-mediated lysis may be dependent not only on the presence of self-peptides or MHC, but also on the high-density expression of specific peptide-MHC complexes. These findings are compatible with the use of an NK-inhibitory receptor with low affinity for the recognition of peptide-MHC complexes. Furthermore, the same peptides were capable of inhibiting lysis of HLA-$A2^+$ T2 cells by NK effectors from both HLA-$A2^+$ and HLA-$A2^-$ donors, indicating that the receptor(s) responsible for this inhibition are expressed independently of HLA-A2 expression in the donors.

These studies were performed using highly enriched (up to 98% purity) NK cells, to exclude a role for T cells in any of the observed effects. We also observed that HER-2 peptides protected targets from lysis by an established NK cell line. In no experiment, though, was complete protection of T2 cells by HER-2 peptides observed. This is not surprising, because the NK cells used in our studies were not clones. It has been shown that different NK clones can respond differently to the same peptide-pulsed targets [8, 9, 23, 25], most likely because of expression of different combinations of inhibitory and activation receptors. Bulk NK populations were used in most of our experiments to mimic more closely the effector/tumor conditions existing in vivo. In fact, it is important to realize that the 15%–30% of tumor cells that might be protected from NK cells by HER-2 peptides would represent a substantial number of malignant cells likely to escape NK cell attack.

Our results show that a side-chain charge at P1 of two different HER-2 peptides is important for protection from lysis. It is interest that the requirement for a specific side-chain in the protection of a target against NK-mediated lysis suggests that certain NK receptors, or

structures on NK cells involved in target lysis, directly contact MHC-bound peptide. Importantly, these effects were observed for the first time when peptides known to induce CTL-mediated lysis in the HLA-A2 system were used. Recent studies have shown sequence-specific NK-potentiating effects for P8 of nonapeptides, although the effects were not associated solely with charged residues at P8 [26]. Furthermore, Peruzzi and collaborators identified a role for P7 and P8 of HLA-B*2705-associated peptides in modulation of NK recognition [28]. Charged side-chains in residues at P7 and P8 in their system enhanced NK-mediated lysis. These studies indicate that residues in certain positions of the class-I-MHC-bound peptides can up- or down-modulate NK lysis. Nevertheless, the effects may be dependent upon HLA type or other unknown factors, which may help explain why one donor of four tested (Fig. 3) in our study showed a somewhat different-from-average pattern of NK inhibition by the C85 variants (inhibition by F1 and G1 but not K1; data not shown). It is most likely that HER-2 peptides were inhibiting lysis directly through the interaction of HLA-A2–peptide complexes with NK receptors, since A2-specific mAb significantly blocked the inhibition. Although the inhibition was not completely blocked when high levels of peptide were used, likely explanations are that monomorphic HLA-A2 was up-regulated more than the MA2.1 conformational epitope or that the antibody was not saturating the HLA-A2 at high peptide concentrations. This could also possibly be due to peptide stabilization of non-classical MHC, such as the deletion variants described by Abu-hadid et al. [1].

Positive stimulation (activation) of NK cells may occur through several different activation or costimulatory receptors on NK cells, such as NKR-P1 proteins, CD16 and CD28, but it appears that the specificity of NK target recognition is often not provided by activation signals, but rather by the presence or absence of inhibitory signals induced by recognition of peptide-MHC complexes [20]. It has been suggested that peptide-induced protection from NK cells may be due to stabilization and/or conformational effects of peptides on MHC class I molecules. However, the role of the peptide in NK recognition is probably not simply to stabilize MHC class I or to promote changes in MHC conformation. NK cells express an array of different receptors that inhibit target cell lysis upon recognition of MHC class I. Examples are the C-type lectin superfamily of receptors (e.g. CD94, NKG2) and the killer-cell-inhibitory receptors of the immunoglobulin superfamily (e.g. p 70, p 58) [4, 20, 29]. Several investigators have now demonstrated that inhibitory receptors on NK cells not only recognize specific types of MHC but also recognize a specific subset of peptides on HLA-B or C [4, 25, 28–30, 43]. Our results in the HLA-A2 system also show that NK cell recognition is sensitive to mutations in peptides that minimally affect monomorphic MHC class I expression. Furthermore, changes in the expression of conformational MHC epitopes did not appear to cause the inhibition of NK-mediated lysis in this model, although such epitopes may have caused increased sensitivity to lysis, as discussed above. It is tempting to hypothesize that NK receptors use a similar mechanism of recognition to the one recently proposed for the T cell receptor [10]; i.e., the proper conformation of the MHC-peptide complex is required for the receptor to "land" on the target, while the changes in side-chain moieties (charge, polarity, van der Waals forces), are responsible for initiation of signaling. This will explain why expression of the MA2.1 conformational epitope does not correlate with recognition, since the epitope recognized by MA2.1 mAb is directly affected by side-chains of residues in pocket A (and possibly B) of HLA-A2, while the BB7.2 mAb detects altered conformation induced by the peptide in a different position (α2 domain, W108), which does not interact directly with peptide side-chains. More extensive studies are needed to address this point, but this study suggests that a number of mutations in peptides (including CTL epitopes) presented by MHC class I may interfere with MHC recognition by NK cells. These findings may have implications for understanding the mechanism by which cells infected with viruses (e.g. influenza or AIDS), and displaying a high rate of mutation, might escape immune defenses. This mechanism may also apply to tumor cells where overexpression of certain gene products (e.g. tyrosinase, gp100, or Muc-1) could lead to the presentation of a high density of self-epitopes with inhibitory effect on NK cells. An additional possibility to be examined is that presentation of mutated peptides (e.g. from p53 or p21 *ras*) may protect tumor cells from NK surveillance.

In support of our conclusions, it has been shown previously that HER-2-overexpressing breast and ovarian cell lines were more resistant to NK-mediated lysis than nonexpressing (or HER-2^{low}) targets [21]. As was the case also in our investigations, resistance in the latter studies could not be attributed solely to an increase in MHC class I or to changes in ICAM-1 expression by the HER-2^{+} targets [11, 22]. Taken together, these results suggest that endogenously processed HER-2 peptides expressed in complexes with MHC class I molecules may contribute to the resistance of HER-2-overexpressing tumor cells to NK-mediated lysis. Therefore, further elucidation of how NK cells recognize peptides may help to explain the aggressiveness of some tumors, as well as provide new insight into the nature of NK cell receptors for antigens.

Acknowledgements The authors would like to thank Timothy W. King for his assistance in the preparation of this manuscript. Peptide synthesis was supported in part by core grant 16672. Supported in part by grants DAMD: 4313 and 7098.

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